Tailoring poplar lignin without yield penalty by combining a null and haploinsufficient CINNAMOYL-CoA REDUCTASE2 allele

Barbara De Meester1,2, Barbara Madariaga Calderón1,2, Lisanne de Vries1,2, Jacob Pollier3, Geert Goeminne3, Jan Van Doorsselaere4, Mingjie Chen5,6, John Ralph5,6, Ruben Vanholme1,2,7 & Wout Boerjan1,2,7✉

Lignin causes lignocellulosic biomass recalcitrance to enzymatic hydrolysis. Engineered low-lignin plants have reduced recalcitrance but often exhibit yield penalties, offsetting their gains in fermentable sugar yield. Here, CRISPR/Cas9-generated CCR2(−/−) line 12 poplars have one knockout CCR2 allele while the other contains a 3-bp deletion, resulting in a 114I115A-to-114T conversion in the corresponding protein. Despite having 10% less lignin, CCR2(−/−) line 12 grows normally. On a plant basis, the saccharification efficiency of CCR2(−/−) line 12 is increased by 25–41%, depending on the pretreatment. Analysis of monoallelic CCR2 knockout lines shows that the reduced lignin amount in CCR2(−/−) line 12 is due to the combination of a null and the specific haploinsufficient CCR2 allele. Analysis of another CCR2(−/−) line shows that depending on the specific CCR2 amino-acid change, lignin amount and growth can be affected to different extents. Our findings open up new possibilities for stably fine-tuning residual gene function in planta.
The lignin polymer provides strength and hydrophobicity to the plant cell wall and is generally derived from the monolignols coniferyl and sinapyl alcohol and low levels of p-coumaryl alcohol. Depending on the plant species, other monomers or derivatives may also contribute to the lignin polymer. After polymerization in the cell wall, the monolignols produce guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units, respectively.

Engineering plants to deposit less lignin is a promising strategy to enable improved biomass processability. However, hurdles need to be overcome for the development of low-lignin elite clones for forestry applications. One hurdle is to reduce lignin amount in a stable way. For example, RNA interference (RNAi) was frequently used to downregulate the expression of lignin biosynthesis genes in poplar. However, this method often results in unstable downregulation of the targeted genes. As an illustration, the red xylem phenotype caused by reductions in CINNAMOYL-CoA REDUCTASE (CCR) activity, appeared in patches on debarked CCR2-downregulated poplar stems, as a consequence of the unequal levels of gene silencing in red versus white regions. Unequal gene silencing levels even appeared between individual clones of the same CCR2-downregulated line.

A second hurdle is to reduce lignin amount without affecting plant development and biomass yield. For example, the CCR2-downregulated poplars with the highest levels of CCR2 down-regulation had up to 24% less lignin and an up to 104% increased enzymatic cellulose-to-glucose conversion without pretreatment. Unfortunately, similar to many other plants yielding higher plant development and biomass yield, for example, the CCR2-downregulated poplars with the highest levels of CCR2 down-regulation had up to 24% less lignin and an up to 104% increased enzymatic cellulose-to-glucose conversion without pretreatment. Unfortunately, similar to many other plants yielding higher cellulose-to-glucose conversion levels, these CCR2-downregulated poplars suffered from a reduction of up to 51% in biomass, entirely offsetting their gains in fermentable sugar yield. Hence, for applications, a method is desired to make plants with a stable and fine-tuned lignin amount to still achieve higher sugar yields in all replicates, but without affecting growth.

To evaluate the specific role of CCR2 in poplar, CCR2(−/−) null mutants are generated using CRISPR/Cas9. In addition to severely dwarfed CCR2(−/−) plants, a biallelically modified line has normal growth. Here, we show that this line, named CCR2(−/−) line 12, contains a knockout and a specific haploinsufficient CCR2 allele (114I115A-to-114T amino-acid change in the corresponding CCR2 protein sequence) that results in a uniformly distributed red xylem phenotype, a 10% reduction in lignin amount and a 25 to 41% increase in saccharification efficiency on a plant basis, depending on the applied pretreatment. Analysis of another CCR2(−/−) line shows that, whereas multiple amino-acid changes in CCR2 can result in lower lignin content (to different extents), they will not all allow normal growth. We propose that in planta screening for combinations of a knockout and a haploinsufficient allele is a promising strategy to fine-tune the desired level of residual gene function.

Results

CCR2(−/−) line 12 grows normally while having red xylem.

To evaluate the effect of fully knocking out CCR2 on the phenotype of poplar, we generated 21 biallelically edited CCR2 mutants in Populus tremula × P. alba by CRISPR/Cas9 using a gRNA (gRNA1) targeting the third exon of the CCR2 gene (Supplementary Fig. 1a). The twenty lines that contained biallelic frameshift mutations in CCR2, CCR2(−/−), lines, were all severely dwarfed (Fig. 1; Supplementary Fig. 1b). Interestingly, one biallelic mutant line did not display observable growth perturbations (Fig. 1). CCR2(−/−) line 12 had a frameshift mutation (1-bp insertion) in the P. tremula CCR2 allele, and a deletion of 3 bp in the P. alba CCR2 allele, which resulted into a substitution of Ile114 and Ala115 for a Thr114 in the corresponding P. alba CCR2 protein sequence (Supplementary Figs. 1b and 2). The amino-acid change occurred in α4 of the CCR2 protein, but not in the active site, NAPD-binding domain, or substrate-binding pocket residues.

To evaluate the growth and xylem phenotypes of CCR2(−/−) line 12, wild type, and CCR2(−/−) line 12 were clonally propagated to generate multiple biological replicates. The replicates were grown in the greenhouse for a period of 20 weeks. Plant height was followed weekly, and by the end of the growth period, the trees were harvested, and biomass parameters were determined. The stem height of CCR2(−/−) line 12 was equal to that of the wild type during the entire growth period (Fig. 2a, Supplementary Table 1). At harvest time, the CCR2(−/−) line 12 plants were morphologically indistinguishable from the wild type (Fig. 2b), and no differences in either stem diameter or fresh and dry stem weight were observed (Table 1).

After debarking the harvested stems, the coloration of the xylem could be judged; whereas wild-type xylem had a white-to-beige color, CCR2(−/−) line 12 xylem displayed a uniform pink-to-red coloration (Fig. 2c). This xylem coloration is often observed in lignin-modified plants and suggested a modified lignin in CCR2(−/−) line 12 as compared to the wild type.

To examine the morphology of the xylem cells in the stem, cross-sections of wild type, CCR2(−/−) line 12, and CCR2(−/−) were observed via light microscopy after Wiesner and Mäule staining, and via fluorescence microscopy (Fig. 3). Wild-type and CCR2(−/−) line 12 stem sections were morphologically indistinguishable. They both had fiber and vessel cells with lignified cell walls, and the vessel cells were open. By contrast, CCR2(−/−) stems showed an overall reduction in lignin deposition in the cell walls of both fiber and vessel cells, and the vessel cells had an irregular shape.
Altered lignin in CCR2(−/−) line 12. To evaluate the lignocellulosic biomass composition of CCR2(−/−) line 12 stems, the cell wall residue (CWR), the cellulose content, and the lignin content and composition of dried debarked stem material were determined (Table 1). CWR was prepared by applying a sequential extraction to remove soluble compounds from the stems. The fraction of CWR (as % of the dry weight) of CCR2(−/−) line 12 did not differ from that of the wild type. The cellulose content of the prepared CWRs was analyzed via the spectrophotometric Updegraff assay which showed that the crystalline cellulose content of CCR2(−/−) line 12 did not differ significantly from that of the wild type. The combined amount of matrix polysaccharides and amorphous cellulose—determined as the mass loss upon trifluoroacetic acid treatment—in the CWRs of CCR2(−/−) line 12 was increased by about 10% when compared to that of the wild type. Next, the fraction of lignin in the prepared CWRs was determined via the Klason and the acetyl bromide methods. Both methods showed that the total lignin amount of CCR2(−/−) line 12 was decreased by about 10% when compared to that of the wild type.

Lignin composition in the CWRs was evaluated via thioacidolysis, an analytical method that quantifies lignin units solely linked by β-O-4 bonds (Table 1). CCR2(−/−) line 12 lignins showed a trend towards releasing more monomers (H+G+S) when compared to wild-type lignins (P = 0.092), indicative for a slightly higher frequency of β-O-4 interunit bonds. In the wild type, H monomers constituted 1.6% of the total identified thioacidolysis-released units. In CCR2(−/−) line 12, the fraction of thioacidolysis-released H units was only 0.3%. The S/G ratio based on thioacidolysis-released monomers was equal for the wild type and CCR2(−/−) line 12. Incorporation of ferulic acid (FA), which is a known minor constituent of lignin, results in the release of three different units after thioacidolysis: β-O-4-FA-I and β-O-4-FA-II are derived from ferulic acid (or ferulate ester) starting units coupled via their O-4 position in β-O-4 interunit bonds, whereas bis-β-O-4-FA is derived from ferulic acid that has undergone a β-O-4 coupling twice at its β-position.  

In agreement with previously reported results for plants deficient in CCR3,5,14–16, the relative abundance of all three
Table 1 Biomass and wall composition of CCR2(−/−) line 12 stems.

|                      | Wild type | CCR2(−/−) line 12 | P value |
|----------------------|-----------|-------------------|---------|
| Fresh weight with bark (g) | 87.6 ± 23.9 | 87.0 ± 19.4 | 0.956   |
| Fresh weight debarked (g) | 57.4 ± 15.0 | 57.0 ± 13.1 | 0.940   |
| Dry weight debarked (g) | 18.8 ± 5.6  | 17.6 ± 4.6  | 0.582   |
| Height (cm)            | 207.4 ± 14.2 | 198.2 ± 11.9 | 0.123   |
| Diameter (mm)          | 11.4 ± 1.0  | 11.0 ± 0.8  | 0.427   |
| CWR (% dry weight)     | 87.8 ± 0.7  | 87.4 ± 0.7  | 0.156   |
| Cellulose (% CWR)      | 39.6 ± 3.8  | 39.5 ± 3.9  | 0.976   |
| Matrix                 | 37.8 ± 1.8  | 41.9 ± 3.11 | 0.002   |
| polysaccharides (%CWR) |           |               |         |
| Total Klason lignin    | 31.1 ± 1.5  | 27.8 ± 0.9** | 5.8 × 10^{-6} |
| (CWR)                  |            |               |         |
| Acid-insoluble Klason lignin (%CWR) | 29.4 ± 1.5 | 26.0 ± 0.9** | 3.4 × 10^{-4} |
| Acid-soluble Klason lignin (%CWR) | 1.69 ± 0.10 | 1.82 ± 0.09 | 0.008   |
| Acetyl bromide lignin  | 17.1 ± 1.3  | 15.4 ± 0.7** | 0.004   |

All values are means ± standard deviation (n = 12 biologically independent samples). Source data are provided as a Source data file.

Plants were grown for 20 weeks in the greenhouse. Stem diameter was determined 3 cm above soil level. At the time of harvest, the height, fresh weight (without leaves; with or without bark), and diameter of the stem were measured. After drying the debarked stems for 2 weeks, the dry weight was determined. Cell wall residue (CWR) was determined after sequential extraction of dry debarked stem material. Crystalline cellulose content was determined by the Updegraff method and the mass loss during TFA extraction was used as an estimate of the amount of matrix polysaccharides. Lignin content was determined by the Klason and acetyl bromide methods. Lignin composition was determined by 2D HSQC NMR and by thioacidolysis. The sum of H, G, and S is expressed in pmol g−1 of Klason lignin. %H/ %G/ %S = 100; other units are mg g−1. 13C HNMR spectroscopy was performed using a Bruker Avance II 300 MHz spectrometer equipped with a cryoprobe and a 5-mm HNMR probe. 1H_13C heteronuclear single-quantum coherence (2D HSQC) spectra were recorded using a homonuclear 1H broadband decoupler, and the spectra were analyzed using TopSpin 3.5 (Bruker). The 2D spectra were acquired in the 400-1200 ppm range for H and 0-200 ppm range for 13C. The spectra were phase and baseline corrected and processed using TopSpin 3.5 (Bruker). The spectra were collected in 8 scans. The spectra were analyzed using TopSpin 3.5 (Bruker). The spectra were collected in 8 scans.

Increased saccharification efficiency in CCR2(−/−) line 12.

Because lignin amount, composition, and polymerization degree greatly influence saccharification yield, we further investigated the saccharification potential of CCR2(−/−) line 12 under conditions of limited saccharification. The cellulose-to-glucose conversion was calculated based on the amount of glucose released upon saccharification of dried debarked stem material after either acidic (1 M HCl, 80 °C, 2 h), alkaline (62.5 mM NaOH, 90 °C, 3 h), or no pretreatment (Supplementary Table 3) and the original cellulose content that was measured for each sample (Table 1). In all three cases, cellulose-to-glucose conversion of biomass from CCR2(−/−) line 12 was significantly higher than that of the wild type (Fig. 4); the cellulose-to-glucose conversion of the non-pretreated samples increased from 23.9% in the wild type to 32.4% in CCR2(−/−) line 12 (i.e., a relative increase of 53%), and after alkaline pretreatment from 30.3 to 46.4% (i.e., a relative increase of 53%) and after alkaline pretreatment from 70.9 to 95.9% (i.e., a relative increase of 35%).

In many plant species, including Arabidopsis, tobacco, and poplar, lowering CCR activity leads to a substantial yield penalty in CCR2(−/−) line 12, which is consistent with the results obtained by thioacidolysis, the ferulic acid marker peak was clearly detected in spectra from CCR2(−/−) line 12 wood, while being absent in spectra from the wild type. In addition, the NMR data enabled the relative measurement of p-hydroxybenzoates that acylate the sidechain γ-OH of G and, predominantly, S units in poplar. The relative frequency of these moieties was increased by 75% in CCR2(−/−) line 12 when compared to the wild type. This observation is in line with the fact that the biosynthesis of p-hydroxybenzoates, in contrast to that of H, G, and S units, is independent of CCR2 activity. The interunit linkage-type distributions were also deduced from the NMR spectra. The lignin of CCR2(−/−) line 12 showed an increase in the relative proportion of β-aryl ether (β-O-4) units, at the expense of resinol (β-β) units. The fraction of phenylcoumarans (β-5) did not differ between CCR2(−/−) line 12 and the wild type.

Finally, analysis by gel-permeation chromatography (GPC) showed that the molecular weight of lignin in CCR2(−/−) line 12 lignin tended to be lower than that of wild-type lignin (Supplementary Table 2).

Together, these data show that, although the growth of the CCR2(−/−) line 12 trees is similar to that of wild-type trees, their wood composition still reflects a deficiency in CCR2.
The twelve individual plants containing monoallelic frameshift mutations in P. alba CCR2 allele (Supplementary Fig. 5b) and will be discussed further below.

The twelve individual plants containing monoallelic frameshift mutations in CCR2 (as shown in Supplementary Figs. 4b and 5b) were grown along with wild type and CCR2(–/–) line 12 in the greenhouse. After a growth period of 11 weeks, both the CCR2(+/–) and CCR2(−/+), monoallelic knock-outs and CCR2(−/–) line 12 plants were equal to the wild type in stem height and diameter, as well as fresh and dry weight (Table 2, in the protein encoded by the mutant P. alba CCR2 allele on total CCR activity was not known. Either the 114I115A-to-114T amino acid change in the P. alba CCR2 protein did not have any effect on the CCR2 activity, in which case the red xylem phenotype and the reduced lignin amount of CCR2(−/–) line 12 can be explained by the haploinsufficiency of the wild-type P. alba CCR2 allele. In this case, the CCR activity of the protein encoded solely by the wild-type P. alba CCR2 allele does not suffice to secure normal lignin biosynthesis. Alternatively, the wild-type P. alba CCR2 allele is haploinsufficient, and the amino-acid change in the mutant P. alba CCR2 protein encoded by CCR2(−/–) line 12 reduces CCR2 activity resulting in the reduced lignin amount and the consequent red xylem phenotype.

To test whether a single CCR2 allele, be it that encoded by the P. tremula or that by the P. alba genome, is sufficient to secure wild-type lignin amount, monoallelic null mutants in CCR2 were generated by CRISPR/Cas9. gRNA2 and gRNA3 were used, targetting the fourth exon of the CCR2 P. alba and P. tremula allele, respectively (Supplementary Fig. 4a, 5a). Six lines contained monoallelic frameshift mutations in P. alba CCR2 (CCR2(+/–); Supplementary Fig. 4b), while six out of eight lines in which the P. tremula CCR2 allele was targeted contained monoallelic frameshift mutations in P. tremula CCR2 (CCR2(−/+) and Supplementary Fig. 5b). The other two lines contained biallelic mutations in CCR2. CCR2(−/–) line 202 contained biallelic frameshift mutations in CCR2 and was severely dwarfed, in agreement with the biallelic frameshift mutants generated previously using gRNA1 (Supplementary Fig. 5b, Figs. 1 and 5). CCR2(−/–) line 206 contained a frameshift mutation (5-bp deletion) in the P. tremula CCR2 allele, and a deletion of 3 bp in the P. alba CCR2 allele (Supplementary Fig. 5b) and will be discussed further below.

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The twelve individual plants containing monoallelic frameshift mutations in CCR2 (as shown in Supplementary Figs. 4b and 5b) were grown along with wild type and CCR2(−/–) line 12 in the greenhouse. After a growth period of 11 weeks, both the CCR2(+/–) and CCR2(−/+), monoallelic knock-outs and CCR2(−/–) line 12 plants were equal to the wild type in stem height and diameter, as well as fresh and dry weight (Table 2,
Fig. 5 Phenotype of poplar containing mono- and biallelic CCR2 mutations. Plants were grown for 11 weeks in the greenhouse. CCR2(+/−) generated via CRISPR/Cas9 using gRNA2, CCR2(−/+) and CCR2(−/−) generated via CRISPR/Cas9 using gRNA3, and CCR2(+/−) and CCR2(−/+) line 12 generated via CRISPR/Cas9 using gRNA1. gRNA1 targets the third exon of both CCR2 alleles whereas gRNA2 and gRNA3 target the fourth exon of the P. alba or P. tremula CCR2 allele, respectively. The status of the biologically independent samples for wild type, monoallelic and biallelic knockout plants (Fig. 6). These observations indicate that the CCR2 allele; the second one that of the P. alba allele; +, wild type; −, knockout; *, protein-modified. The plants shown are representative for seven biologically independent samples for wild type, CCR2(+/−), CCR2(+/+), CCR2(−/−) (gRNA1), and CCR2(−/+). One plant was available for CCR2(−/−) (gRNA3). Scale bar = 10 cm.

Table 2 Biomass and cell wall composition of CCR2(+/−), CCR2(+/+), and CCR2(+/−) line 12 stems.

|                | Wild type | CCR2(+/−) | CCR2(+/+) | CCR2(+/−) line 12 |
|----------------|-----------|-----------|-----------|--------------------|
| Fresh weight (g) | 4.9 ± 0.5 | 3.4 ± 2.6 (0.276) | 5.8 ± 1.6 (0.355) | 4.0 ± 1.1 (0.640) |
| Dry weight (g)   | 1.1 ± 0.2 | 0.8 ± 0.6 (0.389) | 1.4 ± 0.5 (0.530) | 0.9 ± 0.3 (0.528) |
| Height (cm)      | 59.3 ± 2.7 | 51.8 ± 12.6 (0.237) | 64.2 ± 7.5 (0.564) | 53.0 ± 4.2 (0.331) |
| Diameter (mm)    | 6.0 ± 0.0 | 4.8 ± 1.2 (0.052) | 6.4 ± 0.8 (0.674) | 5.5 ± 0.6 (0.521) |
| CWR (% dry weight)| 70.6 ± 4.3 | 75.6 ± 2.0 (0.174) | 73.3 ± 2.6 (0.628) | 74.4 ± 7.5 (0.334) |
| Acetyl bromide lignin amount (CWR) | 16.5 ± 1.2 | 16.2 ± 0.7 (0.888) | 16.7 ± 0.7 (0.936) | 14.0 ± 0.8** (<0.0001) |

Plants were grown for 11 weeks in the greenhouse. Stem diameter was determined 3 cm above soil level. At the time of harvest, the height, fresh weight (without bark and leaves), and diameter of the stem were measured. After drying the debarked stems for 5 days, the dry weight was determined. Cell wall residue (CWR) was determined after sequential extraction of dry debarked stem material. Lignin content was determined via the acetyl bromide method. Lignin composition was determined by thioacidolysis. The sum of H, G, and S is expressed in μmol per g acetyl bromide lignin. %H = %G + %S. Other units are expressed versus H + G + S. The data represent means ± standard deviation (for biomass measurements: wild type, CCR2(+/−) and CCR2(+/+), n = 6 biologically independent samples; CCR2(+/−) line 12, CCR2(+/−), CCR2(+/−), n = 7 biologically independent samples; for cell wall analysis: wild type and CCR2(+/−) line 12, n = 7 biologically independent samples, CCR2(+/−) and CCR2(+/−), n = 6 biologically independent samples). **P < 0.01; one-way ANOVA with Dunnett’s post hoc test; the exact P value (for the pairwise comparison with the wild type) is shown between parentheses in the table. The status of the CCR2 alleles present in P. tremula × P. alba is denoted between the parentheses; the first one represents that of the P. tremula allele, the second one that of the P. alba allele; +, wild type; −, knockout; *, protein-modified. Source data are provided as a Source data file.

Fig. 5. After debarking the harvested stems, the typical red coloration of the xylem was seen to be present in CCR2(−/−) line 12, but absent in the wild-type and the CCR2 monoallelic knockout plants (Fig. 6). These observations indicate that the lignin of the CCR2 monoallelic knockout plants resembles that of the wild type and not that of CCR2(−/−) line 12. To validate this, the lignin content and composition of dried debarked stem material were determined (Table 2). The acetyl bromide lignin content in the CWR from CCR2 monoallelic knockout plants was equal to that of the wild type, whereas that of the CCR2(−/−) line 12 was reduced by ~15%. In agreement with the normal xylem coloration, no significant differences in thioacidolysis-released aromatic units were observed between the CCR2 monoallelic knockout plants and the wild type. Similar to the results of 20-week-old stems, 11-week-old CCR2(−/−) line 12 stems had a decreased frequency of H monomers and an increased frequency...
proliferant (which explains 53% of the variation), the metabolic changes of (P. tremula) features) (Supplementary Data 1). PCA showed that, according to the peaks (mass-to-charge ratio [m/z] UHPLC-MS) on debarked stems of wild type, CCR2 were found to have a higher intensity in independent samples per line. Scale bar = 1 cm.

of all three thioacidolysis-released ferulic acid units when compared to the wild-type and CCR2 monoallelic knockout plants.

Judged by the normal growth, lignin amount and lignin composition of the CCR2 monoallelic knockout plants, the P. tremula and the P. alba CCR2 alleles appear to both be haplosufficient in P. tremula × P. alba.

Phenolic profiling of the different CCR2 mutants. To further examine the haplo(in)sufficient status of the CCR2 alleles in P. tremula × P. alba, and investigate to what extent the metabolic changes of CCR2(−/−) line 12 reflect those expected from CCR2 deficiency, comparative phenolic profiling was performed via ultra-high-pressure liquid chromatography-mass spectrometry (UHPLC-MS) on debarked stems of wild type, CCR2(+/*), CCR2(+/−), CCR2(−/+), CCR2(−/−) line 12, and CCR2(−/−). Principal component analysis (PCA) was performed on a total of 6182 peaks (mass-to-charge ratio [m/z] features) (Supplementary Data 1). PCA showed that, according to the first principal component (which explains 53% of the variation), the metabolic profiles of wild type, CCR2(+/*), and CCR2(−/−) were indistinguishable, whereas those of CCR2(−/*) line 12 were situated between those of CCR2(−/−) and wild type (Fig. 7a). The second principal component, which explains 10.1% of the variation, reflects variation within the genotypes (and not between the genotypes) and can be attributed to biological and/or technical variation. Next, univariate statistical analysis was applied to the selected peaks to screen for peaks with significantly different intensities in CCR2(+/−), CCR2(+/−), CCR2(−/*) line 12, or CCR2(−/−) mutants compared with their levels in wild-type plants. After applying specific filters (see "Methods"), no significant differences were found between either CCR2(+/−) or CCR2(+/−) and the wild type (Fig. 7b), again showing that the P. tremula and the P. alba CCR2 alleles are both haplosufficient in P. tremula × P. alba. The reduction in lignin amount in CCR2(−/*) line 12 is therefore not solely a consequence of the P. tremula CCR2 null allele.

By contrast, using the same specific filters, 960 and 1815 peaks were found to have a higher intensity in CCR2(−/*) line 12 and CCR2(−/−), respectively, when compared to the wild type, of which 800 were in common (Fig. 7c, d). In addition, 258 and 1854 peaks had a lower intensity in CCR2(−/*) line 12 and CCR2(−/−), respectively, when compared to the wild type, of which 199 were in common. Next, the top 10 highest peaks with significantly higher and lower intensities in CCR2(−/*) line 12 were structurally characterized based on their mass-to-charge ratio (m/z), retention time, and tandem mass spectrometry (MS/MS) (Supplementary Figs. 6–11, Supplementary Table 4). The ten highest peaks with significantly higher intensities in CCR2(−/*) line 12 could be assigned to ten compounds, of which seven could be (partially) structurally characterized as conjugates of ferulic acid, vanillic acid, syringic acid, and caffeic acid, and three remained unknown. The ten highest peaks with significantly lower intensities in CCR2(−/*) line 12 could be assigned to nine compounds, of which five could be structurally characterized as oligolignols, and four remained unknown.

The relatively high fraction of differential peaks that CCR2(−/*) line 12 shares with CCR2(−/−) (Fig. 7d, Supplementary Table 4), and the identities of the differential compounds in CCR2(−/*) line 12, which are in line with the position of CCR2 in the lignin biosynthetic pathway (Supplementary Table 4), again underline the haploinsufficiency of the mutant P. alba CCR2 allele in CCR2(−/*) line 12.

A haploinsufficient P. alba CCR2 allele in CCR2(−/*) line 12. In CCR2(−/*) line 12, the P. alba CCR2 protein sequence differs in only two amino acids from the wild-type P. alba CCR2 protein sequence (Supplementary Fig. 2). Because the analysis of monoallelic CCR2 mutants suggested that the wild-type CCR2 alleles are haplosufficient in P. tremula × P. alba (Table 2, Fig. 7), the reduced lignin content of CCR2(−/*) line 12 is probably the consequence of the mutation in the P. alba CCR2 allele (in combination with the null P. tremula CCR2 allele), which might encode an enzyme with a lower CCR activity or a lower protein stability as compared to the wild-type P. alba CCR2-encoded enzyme. To validate this, yeast assays were performed in which the activities of the wild-type and the mutant P. alba CCR2 proteins were investigated based on the production of coniferaldehyde from feruloyl-CoA, the substrate of CCR2.

As it was not possible to feed feruloyl-CoA to the yeast cultures, whereas it was possible to feed ferulic acid, we created yeast strains that express 4-coumarate:CoA ligase (4CL). The 4CL enzyme converts ferulic acid to feruloyl-CoA, allowing the CCR2 enzyme activities to be tested (Fig. 8a). All yeast cultures were fed with ferulic acid and extracts were analyzed by GC-MS. Yeast cultures expressing only 4CL produced no coniferaldehyde upon feeding with ferulic acid (Fig. 8b). However, upon co-expression of 4CL and wild-type P. alba CCR2, coniferaldehyde was formed (peak 1, Fig. 8b; Supplementary Fig. 12a, b). In addition to coniferaldehyde, two other metabolites, which were identified based on their EI-MS spectra as dihydroconiferyl alcohol and coniferyl alcohol (peak 2 and peak 3, respectively), accumulated in this strain (Fig. 8b; Supplementary Fig. 12c, d). As these two metabolites also accumulated in yeast cultures expressing only 4CL and additionally fed with coniferaldehyde (Fig. 8b), we concluded that yeast cells further metabolize coniferaldehyde to dihydroconiferyl alcohol and coniferyl alcohol. Therefore, these two metabolites can be used as additional diagnostic markers for the production of coniferaldehyde in yeast cultures.

In contrast to yeast cultures expressing both 4CL and the wild-type CCR2, yeast cultures expressing 4CL and the mutated P. alba CCR2 failed to produce coniferaldehyde, dihydroconiferyl alcohol, and coniferyl alcohol (Fig. 8b). Based on these data, we concluded that, in yeast, no detectable enzymatic activity was present for the mutant P. alba CCR2 protein. The normal growth but reduced lignin content of CCR2(−/*) line 12 suggests that, in planta, the mutant P. alba CCR2 protein had a reduced CCR
Fig. 7 Phenolic profiling of CCR2(+/−), CCR2(−/+), CCR2(−/*) line 12, and CCR2(−/−) stems. Plants were grown for 11 weeks in the greenhouse. a Plot of principal component 1 (PC1) and PC2 of principal component analysis (PCA) on 6182 peaks. Black dots, wild type; blue dots, CCR2(+/−); cyan dots, CCR2(−/+); gray dots, CCR2(−/*) line 12; red dots, CCR2(−/−). b, c Volcano plots visualizing the differences between b wild type and CCR2(+/−) or CCR2(−/+), and c wild type and CCR2(−/*) line 12 or CCR2(−/−). Magenta and black dots represent peaks that are different and not different in intensity, respectively, based on the filters: fold change (FC) > 2 and P value <0.01 (one-way ANOVA with Dunnett’s post hoc test). d Venn diagrams of the number of peaks with significantly differential intensities (magenta dots from the volcano plots in (c)) between wild type and either CCR2(−/*) line 12 or CCR2(−/−). Wild type, n = 6 biologically independent samples; CCR2(−/*) line 12, n = 6 biologically independent samples; CCR2(−/−), n = 5 biologically independent samples.
activity and/or reduced protein stability as compared to the wild-type \textit{P. alba} CCR2 protein, but did not fully lose its activity, as this would lead to dwarfism as observed in the CCR2\text{	extasciitilde}/\text{	extasciitilde} knockout mutants. The apparent null-activity in yeast might be explained by the lack of the proper cellular context of a normal lignifying cell, such as pH, interaction, and/or stabilization partners, etc., which might influence CCR2 enzymatic activity.

**Fig. 8 CCR2 activity assays in yeast.** The relative activity of the mutant \textit{P. alba} CCR2 protein (as present in CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12) was determined in yeast. \textbf{a} Principle of the yeast feeding assay. Yeast cultures were engineered to express 4CL and the wild-type \textit{P. alba} CCR2 protein or mutated \textit{P. alba} CCR2 protein (as present in CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12). After feeding the yeast cultures with ferulic acid, the activity of the respective CCR2 protein was judged based on the production of coniferaldehyde (the product of CCR2, peak 1), coniferyl alcohol (peak 2), and dihydroconiferyl alcohol (peak 3). See Supplementary Fig. 12 for the spectra of peaks 1-3. \textbf{b} GC-MS chromatograms of an authentic coniferaldehyde standard and extracts from ferulic acid-fed yeast cells expressing 4CL in combination with either an empty vector (EV), mutant \textit{P. alba} CCR2 or wild-type \textit{P. alba} CCR2. The results shown are representative of five biologically independent samples.

**Not all CCR2\text{	extasciitilde}/\text{	extasciitilde} lines display a normal growth phenotype.** Using gRNA3 targeting the fourth exon of CCR2, another CCR2\text{	extasciitilde}/\text{	extasciitilde} line was obtained having an indel pattern in the CCR2 alleles that was similar to that present in the CCR2 alleles of CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12 (albeit in a different position of the CCR2 gene). CCR2\text{	extasciitilde}/\text{	extasciitilde} line 206 contained a frameshift mutation (5-bp deletion) in the \textit{P. tremula} CCR2 allele, and a deletion of 3 bp in the \textit{P. alba} CCR2 allele (Supplementary Fig. 5b). The latter resulted in a substitution of Trp175 and Asp176 for a Tyr175 in the corresponding \textit{P. alba} CCR2 protein sequence (Supplementary Fig. 13). The amino-acid change occurred in a6 of the CCR2 protein, but not in the active site, NAPD-binding domain, or substrate-binding pocket residues9–11.

CCR2\text{	extasciitilde}/\text{	extasciitilde} line 206 had a lignin amount and growth that was increased when compared to that of CCR2\text{	extasciitilde}/\text{	extasciitilde}, but still severely reduced when compared to that of the wild type (Supplementary Fig. 14a, b, d). However, similar to CCR2\text{	extasciitilde}/\text{	extasciitilde} stems, CCR2\text{	extasciitilde}/\text{	extasciitilde} line 206 stems also displayed a red xylem coloration and collapsed vessels (Supplementary Fig. 14c, d).

**Discussion**

In previously generated CCR2-downregulated poplars, the red xylem phenotype appeared in patches, even among clones originating from the same plant, as a consequence of unstable downregulation3. A similar observation was made for poplars with RNAi-mediated downregulation of 4\textit{CL}14. In contrast, CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12 poplars had a stable reduction in lignin amount along the stem and in all biological replicates, as judged from the uniformly distributed red xylem phenotype, which is a big step forward in achieving stably modified lignocellulosic biomass.

After alkaline pretreatment, the cellulose-to-glucose conversion increased from 70.9\% in wild type to an almost complete conversion (95.9\%) in CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12. Also without pretreatment, CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12 had a 36\% higher cellulose-to-glucose conversion than the wild type. Since no biomass penalty was observed for CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12, even on a plant basis, this line yielded substantially more sugar than the wild type upon limited-saccharification experiments. However, before further translation of this knowledge to generate feedstock for the biorefinery, CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12 poplars remain to be evaluated for growth and lignin amount when cultivated in the field, where they are exposed to different biotic and abiotic stresses. Indeed, it has been shown that genetically modified trees that grow normally in the greenhouse do not always grow as well as wild type when grown in the field20. But even if CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12 plants would have a yield penalty in the field, the specific mutation present in the \textit{P. alba} CCR2 allele of CCR2\text{	extasciitilde}/\text{	extasciitilde} line 12 might still be valuable to engineer lignin in another cultivar or crop (such as Eucalyptus),
because the effect of a mutation on the phenotype depends not only on the environment, but also on the specific genetic background in which it resides\(^{33,34}\).

Typically, using CRISPR/Cas9, knockout lines are generated. However, knockout mutants in lignin biosynthesis frequently suffer from undesired phenotypes such as dwarfish\(^{35-38}\). Lignin levels in plants can be reduced without affecting plant growth, but if lignin drops below a critical level, effects on vessel architecture and growth become apparent\(^{39,40,41}\). For example, for CCR2 downregulated (RNAi-mediated) poplars, a range of plants with different levels of CCR2 downregulation and growth was obtained (even amongst biological replicates originating from the same plant\(^{35}\)). Approximately 5% of all CCR2-downregulated lines that have been generated displayed severe dwarfism and could only survive in tissue culture\(^{35}\). These lines were most likely the plants with the highest reduction in CCR2 activity. Of the CCR2-downregulated plants that survived on soil, the plants with the highest amount of red coloration (and thus the lowest amount of CCR expression and lignin amount) had the highest increase in saccharification efficiency, but also showed collapsed vessels and suffered some biomass yield penalty\(^{35,36}\). Stem levels lower than 5% of the normal level of lignin and biomass yield when compared to CCR2(-/-) but still displayed collapsed vessels, whereas CCR2(-/-) line 12, with its mild reduction in lignin amount compared to the wild type, displayed normal vessels and growth, even in clonally replicated trees. As the wild-type CCR2 alleles are both haplosufficient in P. tremula x P. alba, the differences in lignin amount and growth between CCR2(-/-) line 206 and line 12 could be attributed to the 3 bp deletions occurring in the fourth and third exon of P. alba CCR2 of CCR2(-/-) line 206 and line 12, respectively (see Supplementary Note 1 for an explanation of the impossibility of the off-target effect).

Our research shows that generating a range of allelic variants in CCR2, specific gRNAs (targeting either the P. tremula or the P. alba CCR2 allele) were selected based on the criteria described above. The best suitable gRNA sequences were GTGTTATTT CGTGGAAAGG (gRNA2) and GGAACAAAGCTGTACGGA (gRNA3).

To introduce monoallelic mutations in CCR2, specific gRNAs (targeting either the P. tremula or the P. alba CCR2 allele) were selected based on the criteria described above. The best suitable gRNA sequences were GTGTTATTT CGTGGAAAGG (gRNA2) and GGAACAAAGCTGTACGGA (gRNA3).

For genotyping the regenerated, transformed shoots, the following primers pairs (designed using the Primer3 0.4.0 software) were used for PCR for identifying the mutations present in the CCR2 alleles. For the PCR using the DNA extracted from P. alba 717-1B4, primers were designed with the construct containing gRNA1: forward 5′-TGACTGGATTATTGCTGG-3′, reverse 5′-GATACTCTGGTGCTCTG-3′; for gRNA2: forward 5′-AGCTTGGCCGTGTTGTTCTG-3′, reverse 5′-CGGTGAGGACTCTTGG-3′; for gRNA3: forward 5′-ACCCTCGTCTGATCGT-3′, reverse 5′-GGAAGCGTCTCAAAGACT-3′. The PCR products were sequenced by Eurofins (Eurofins Genomics) and analyzed via CLC Main Workbench.

The wild-type controls (P. tremula x P. alba 717-1B4) originate from the same batch of plants that was used to generate the transgenic lines. However, instead of generating callus-tissue and performing Agrobacterium-mediated transformation, the wild-type controls were maintained and clonally propagated to serve as a control for the regenerated, transformed shoots.

For clonal propagation, 4 plants were grown for 3 to 4 months in tissue culture (on M1 medium as described below) under long-day conditions (16-h light and 8-h dark photoperiod, 24°C) were used. More specifically, the stems were cut into pieces of ≤5 cm and put on fresh M1/2 medium to allow the development of roots and new shoots.

### Media used for plant cultivation

- **M**: Murashige and Skoog basic medium (Duchefa) 4.4 g per liter, Morrel and Wetmore vitamins 10 ml per liter, L-cystein 1 mg per liter, L-glutamine 200 mg per liter, sucrose 30 g per liter, plant agar (Duchefa) 6.2 g per liter.
- **M1**: as M but with half-strength macro-nutrients, sucrose 20 g per liter, IAA 5 μM.
- **M1**: as M but with NAA 10 μM and 2iP 5 μM.
- **M2**: as M but with carbenicillin 500 mg per liter, cefotaxime 250 mg per liter.
- **M3**: as M but with carbenicillin 500 mg per liter, cefotaxime 250 mg per liter, thidiazuron 0.1 μM.

### Plant growth and harvest

After growing for 4 months in tissue culture under long-day conditions, the transgenic poplars and their wild-type controls were transferred to soil. More specifically, the poplars were transferred to pots of 5.5 cm diameter filled with Saniflor commercial soil (Van Israel nv), placed in a tray filled with water, and covered with a cage liner (Tecniplast APET disposable cage liner for cage body 1291H) for acclimatization. After 2 weeks, one side of the cage liner was lifted 1 cm above water level and kept accordingly for 1 day, after which the whole cage was lifted. The next day, the cage liner was removed and the acclimatized plants were transferred to bigger pots filled with a Saniflor commercial soil (Van Israel nv) (10 liter). Note that CCR2(-/-) plants were found to be extremely sensitive to the adjustment to greenhouse conditions. Therefore, here, the cage liner was kept closed for a period of 5 weeks and slowly opened over a period of 3 weeks to allow a much slower transition to the less-humid greenhouse conditions. Even with this precautionary measure, only a small number of CCR2(-/-) poplars recovered, whereas all plants of the other genotypes survived.

In total, six batches of plants were grown for 11 or 20 weeks in the greenhouse under a 16-h light and 8-h dark photoperiod at 21°C. The first batch, containing wild-type, the 20 individual CCR2(-/-) lines, and CCR2(-/-) line 12, was grown for 11 weeks in the greenhouse and used for a picture of the growth phenotype (Fig. 1).

The second batch, containing wild type and CCR2(-/-) line 12, was grown for 20 weeks in the greenhouse. The height of the trees was determined weekly (Fig. 2a, b). After that, the diameter of the stems was determined (3 cm above soil level) (Table 1). Next, the stems were harvested (40 cm above soil level), the leaves were removed from the stem and the fresh weight of the stems was determined before

### Methods

**Plant material and vector construction.** To introduce biallelic mutations in CCR2, a list of 30 protospcers with the N20-NGG motif specific for the P. tremula x P. alba CCR2 alleles (Potri.003G181R90) was extracted from the Aspen database (http://aspendb.uga.edu)\(^ {12,14}\). Next, the possible protospcers were analyzed based on their position in the CCR2 alleles and the possible off-targets via the Aspen database\(^ {23,24}\). In addition, GC-content and absence of a TTTT TT sequence were considered. Based on these parameters, the most suitable gRNA sequence was chosen: GCAAAATAATGTGCATGGG (gRNA1). gRNA1 targets the third exon of both CCR2 alleles. Using the pUC gRNA Shuttle (Addgene plasmid #47024) plasmid, gRNA1 was cloned into the p201N-Cas9 plasmid (Addgene plasmid #9175) by Gibson assembly\(^ {25}\). The p201N-Cas9 gRNA_CCR2 vector was transferred into Agrobacterium tumefaciens strain C58C1 pMP90 by electroporation. Agrobacterium-mediated transformation of P. tremula x alba 717-1B4 was performed via co-cultivation\(^ {26}\). For this, 40 explants (which were preincubated on solidified M1 medium (see below) for 48 h at 24°C in the dark) were dipped into 25 ml of Agrobacterium solution (grown on M liquid medium (see below) until reaching a concentration of 5 x 10⁶ cfu per ml) and slowly stirred for 16 h. After spotting on sterile paper, the explants were placed on solidified M1 medium for 48 h. Subsequently, the explants were washed with tetracycline solution (25 mg per liter) and sterile water, and transferred onto M2 medium (see below) for 10 days at 24°C. Transferring the explants to solid M3 medium (see below) with 100 mg per liter kanamycin. After growing for approximately 20 days in standard light conditions, the plants (that developed roots and elongated shoots) were micropropagated on M1/2 medium (see below) with 50 mg per liter kanamycin.

The first batch, containing wild-type, the 20 individual CCR2(-/-) lines, and CCR2(-/-) line 12, was grown for 11 weeks in the greenhouse and used for a picture of the growth phenotype (Fig. 1).

The second batch, containing wild type and CCR2(-/-) line 12, was grown for 20 weeks in the greenhouse. The height of the trees was determined weekly (Fig. 2a, b). After that, the diameter of the stems was determined (3 cm above soil level) (Table 1). Next, the stems were harvested (40 cm above soil level), the leaves were removed from the stem and the fresh weight of the stems was determined before
and after removing the bark (Table 1). The dry weight of the debarked stems was determined after air-drying the stems for 2 weeks at ambient temperature (Table 2). The dried debarked stem was ground in a ball mill for cell wall analysis and saccharification assays (Table 1, Fig. 4, Supplementary Tables 2 and 3).

The third batch consisted of the same lines present in batch 2 (wild type and CCR2 (−/−)) line 12, but now also accompanied by CCR2 (−/−) mutants. This batch was grown for 20 weeks in the greenhouse. The height of the trees was determined after the trees had grown for 20 weeks.

Microscopy. Fifteen-micrometer-thick stem slices were made using a Reichert-Jung 2040 Autocut Microtome (Leica). Sections were stained with Wiesner and Mäule reagents; Wiesner staining was performed by adding a drop of 1% Mäule reagents; Wiesner staining was performed by adding a drop of ammonium hydroxide solution. Sections were then fixed in a mixture of concentrated HCl (37 M), followed by the addition of concentrated ammonium hydroxide solution.

Cell wall characterization. To determine the crystalline cellulose amount, the Updegraff method was used on 5 mg of CWR stem powder suspended in 0.6 mL DMSO-d6:pyridine-d5 (4:1, v/v). The acid-soluble lignin concentrations were calculated by means of the Bougouin–Lambert–Beer law.

For the lignin composition determination via thioacidolysis, 15 mg of CWR was weighed into a 5-mL glass Wheaton vial with Teflon-lined screw-cap. First, the samples were incubated with 1 mL reaction mixture (containing of 2.5% boron trifluoride etherate and 10% ethanethiol in dioxane) for 4 h at 98 °C, while shaking at 130 °C. After cooling down to 20 °C, the samples were treated with 0.2 mL tetracosane in dichloromethane (5 mg per mL) and 0.3 mL of 0.4 M sodium bicarbonate was added. Next, the samples were extracted with 2 mL milliQ water and 1 mL dichloromethane. Using a Pasteur pipette packed with a small cotton plug and a spatula-point of anhydrous sodium sulfate, the crude fraction was filtered and dried under vacuum. The samples were dried under vacuum and resuspended in 0.2 mL dichloromethane. Derivatization occurred by adding 20 µL pyridine and 100 µL N-O-bis(trimethylsilyl) acetamide to 20 µL of resuspended sample and incubating the samples for 2 h at 25 °C while shaking at 750 rpm. The reaction product was analyzed with GC-MS. GC-MS analysis was carried out using a 7890B GC system equipped with a 7693A Automatic Liquid Sampler and a 7250 Accurate-Mass Quadrupole Time-of-Flight MS system (Agilent Technologies). One microliter of the reaction product was injected in splitless mode with the injector port set to 280 °C. Separation was achieved with a VF-5ms column (30 m × 0.25 mm, 0.25 µm; Varian CP9013; Agilent Technologies) with helium carrier gas at a constant flow of 1.2 mL per min. The oven was held at 130 °C for 5 min post-injection, ramped to 200 °C at 10 °C per min, ramped to 250 °C at 3 °C per min, held at 250 °C for 5 min, ramped to 320 °C at 20 °C per min, held at 320 °C for 5 min, and finally cooled to 130 °C at 50 °C per min at the end of the run. The MSD transfer line was set to 280 °C and the electron ionization energy was 70 eV. Full-MS spectra were recorded between m/z 50 and 800 at a resolution of 25,000 and with a solvent delay of 10.0 min. Peak integrations for quantification of the lignin monomers were carried out using the MassHunter Quantitative Analysis (for QTOF) package (Agilent Technologies).

The lignin composition determination via NMR was performed on 50–50 mg of ground stem powder suspended in 0.6 mL DSMO-d4-pyridine-d4 (4:1, v/v). The samples were sonicated, with occasional mixing by vortexing, until a uniform gel was formed. NMR experiments were performed on a Bruker Biospin (Billerca) Avance 700 MHz spectrometer equipped with a 5-mm QH 1H/13C cryoprobe with inverse geometry (protic solvent closest to the sample). As in internal reference, the central DSMO solvent peak was used (δc, 39.5, δh, 2.49 ppm). The 1H–13C correlation experiment was an adiabatic HMQC experiment (Bruker standard pulse sequence ‘hsqctgsp22p2’; phase-sensitive gradient-edited-2D HSQC using adiabatic pulses for inversion and refocusing). The parameters used for the HMQC experiments were a 1.5 s delay from F1 to F2, 1024 (F2) 1024 (F1) points with 13598 data points (acquisition time, 100 ms) and 200 to 0 ppm in F1 (13C) with 570 increments (F1 acquisition time, 8 ms) of 32 scans with a 1 s interscan delay. The δc delay was set to 0.86 ms (1/18, J = 145 Hz). The total acquisition time for a sample was ∼5 h. Processing used typical matched Gaussian apodization (GB = 0.001, LB = 0.001, 1H/13C in F2 and Gaussian apodization (GB = 0.001, LB = 0.03) also in F1 (without using linear prediction). Volume integration of contours in HSQC plots used TopSpin 4.0.8 software, and no correction factors were used.

**Analytical gel-permeation chromatography.** Ground powder (150 mg) was treated with cellulase (5000 U/mg in biomass) in sodium acetate buffer (pH 5.0, 40 mL) at 37 °C for 72 h. After centrifugation, the precipitate was collected and washed five times with deionized water to obtain enzyme lignin for gel-permeation chromatography (GPC) analysis.

Enzyme lignin (10–15 mg) was dissolved in dimethylformamide (DMF)/lithium bromide (Lith) solution (700 µL, 0.1 M Lith) and filtered through a syringe filter (0.45 µm, PVDF). GPC analysis was performed utilizing a Shimadzu LC20-AD LC pump equipped with a Shimadzu SPD-M20A UV-vis detector set at 280 nm and a
Shimadzu RID-10A refractive index detector. The GPC column set consisted of 4 TOSOH (TOSOH Biotec, LLC) GPC columns and a guard column (TSKgel Guard Alpha 6.0 mm ID × 4.0 cm, 13 μm − TSKgel Alpha-M 7.8 mm ID × 30 cm, 13 μm − TSKgel Alpha-M 7.8 mm ID × 30 cm, 13 μm − TSKgel Alpha-M 7.8 mm ID × 30 cm, 13 μm). The column oven was held at 50°C during analysis. The mobile phase was DMF with 0.1 M LiBr, the flow rate was 0.5 mL per min, and the oven temperature was 40°C on an Agilent E1100 (Agilent Technologies, Santa Clara, CA, USA). Molecular ions were detected using Shimadzu GPC postrun software via a conventional calibration curve using a ReadyCal polystyrene Kit (Sigma-Aldrich, Aldrich # 76552, Mp) 250-70000).

Saccharification assay. Saccharification was performed on 10 mg of dried, ground stem material. The samples were saccharified for 72 h using no pretreatment, acidic pretreatment (1 M HCl, 80°C for 2 h while shaking at 750 rpm), or alkaline pretreatment (62.5 mM NaOH, 90°C for 3 h while shaking at 750 rpm). After the pretreatment, the samples were centrifuged for 5 min at 10,080 × g. The pellet was washed three times with 1 mL milliQ water, and incubated in 1 mL of 70% ethanol for 16 h at 55°C. After another centrifugation step (5 min at 10,080 × g), the pellet was washed three times with 1 mL 70% ethanol and one time with 1 mL acetonitrile, centrifuged for 5 min at 10,080 × g, 10,000 rpm, dried under vacuum, and weighed.

The enzyme mix consisted of a 5:3 ratio of cellulases from Trichoderma reesi ATCC 26291 and β-glucosidase (Novozyme) which were first desalted over an EconoPac 10 DG column (Bio-Rad), stacked with Bio-gel® P-6 DG gel (Bio-Rad) according to the manufacturer’s guidelines. The activity of the enzyme mix was measured with a filter paper assay and was 0.18 FPU (filter paper units) per mL. Subsequently, the samples were dissolved in 1 mL acetic acid buffer solution (pH 4.8) and incubated at 750 rpm. After 5 min of incubation, the samples were mixed with freshly prepared enzyme mix was added. After spinning down the samples in a benchtop microcentrifuge, 20 μL of the supernatant was taken after 72 h of incubation at 50°C and 30-fold diluted with acetic acid buffer (pH 4.8). To determine the concentration of glucose in these samples, a spectrophotometric color reaction was used (glucose oxidase, glucose oxidase (GOPOD), Sigma) containing 50 mg 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 173 μL 1 M LiBr, the resulting reaction mixture contained 50 μg GOD, and 173 μL 0.1 M LiBr, the mixture was incubated at 55°C for 30 min. The absorbance was read at 414 nm using a microplate reader (Molecular Devices). The absorbance of the samples was normalized to the absorbance of the set blank.

Phenolic profiling. For phenolic profiling, the frozen debarked stem parts were cut into small pieces using scissors. Subsequently, the stem pieces were extracted by adding 1 mL methanol and incubating for 15 min at 70°C while shaking at 1000 rpm. After centrifugation at 19,757 × g, the supernatant was taken after 72 h of incubation at 50°C and 30-fold diluted with acetic acid buffer (pH 4.8). For phenolic profiling, the sample was derivatized by using 10 μL pyridine and 50 μL N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) for GC-MS analysis on a GC model 6890 and MS model 5973 (Agilent Technologies). One microliter of the sample was splitless and injected into the GC system with a column (Waters), while maintaining the temperature at 80°C. A gradient of two buffers (A and B) was used: buffer A (99:1:0.1 water: acetonitrile/formic acid, pH 3) and buffer B (99:1.0 acetonitrile/water/formic acid, pH 3), as follows: 99% A for 0.1 min decreased to 50% A in 30 min, to 0% from 30 to 40 min. The flow rate was 0.35 mL per min, and the injection volume was 1 μL. This UHPLC system was connected to a Vion IMS TOF hybrid mass spectrometer (Waters), The LockSpray ion source was used in negative electrospray ionization mode under the following specific conditions: capillary voltage, 3 kV; reference capillary voltage, 2.5 kV; cone voltage, 30 V; source offset, 50 V; source temperature, 120°C; desolvation gas temperature, 550°C; desolvation gas (nitrogen), 800 L/min per h; and cone gas flow, 50 L/min per h. The electron ionization energy for full MS was set at 70 eV (low energy) and ramped from 20 to 70 eV (high energy), intelligent data capture intensity threshold was set at 3. For negative mode, a solvent delay of 7.8 min. GC-MS data were recorded and visualized with Agilent software and visualized via the MSDS software (Version 2.6, NIST).

Statistical analyses. MS Excel 2016 and SAS 9.4 were used for statistical analysis. The specific method used is mentioned in the respective Table and Figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. Sequences data that support the findings of this study were obtained from the Aspen database (for the CCR2 sequences from P. tremula × P. alba; https://www.ncbi.nlm.nih.gov/nuccore/XM_008365460), or NCBI (for the Malus domestica 4CL sequence; https://www.ncbi.nlm.nih.gov/nuccore/XM_008365460), or reported in Supplementary Information file. Source data are provided with this paper.

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Author contributions
B.D.M., R.V., and W.B. designed the research; B.D.M., B.M., J.P., L.d.V., J.V.D., and M.C. performed the experiments; B.D.M., B.M., J.P., L.d.V., and J.R. performed the data analysis; B.D.M., R.V., and W.B. wrote the article with contributions from all authors.

Competing interests
A patent application (WO2019234141A1) stating B.D.M., R.V., and W.B. as inventors on the use of plants having weak CCR alleles, resulting in lower lignin amounts and increased saccharification, has been filed jointly by VIB and Ghent University. All other authors declare no competing interests.

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Correspondence
and requests for materials should be addressed to W.B.

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