MODIFYING LIGNIN TO IMPROVE THE UTILITY OF *POPULUS* AS A BIOENERGY CROP

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As a perennial woody plant, hybrid poplars (species within the genus *Populus*) offer several advantages as a bioenergy crop, including rapid growth rates, the ability to cycle nutrients, a wide geographic distribution, genetic diversity, amenability to genetic engineering, abundant genomic resources, greater flexibility with regard to harvest, and more efficient transport and storage. The phenolic cell-wall polymer lignin constitutes a significant barrier to biomass conversion for all cellulosic feedstocks; however, it is also essential to normal plant growth and development. Recent advances in our understanding of how lignin is synthesized provide options for modifying the composition of lignin, in an effort to improve conversion efficiencies. In poplar, we have over-expressed various genes involved in the lignin biosynthetic pathway, and used RNA interference (RNAi) to down-regulate others.

Recently, we have begun testing the maize *Corngrass1* (*Cg1*) gene, which encodes a unique MIR156-class microRNA, in poplar. In herbaceous species, *Cg1* overexpression fixes plant development in the juvenile phase and, thus, affects the initiation of meristems and lateral organs. Plants in which it has been expressed constitutively produced multiple axillary branches, grew faster, contained less lignin, and were either sterile or exhibited delayed flowering. We have over-expressed *Cg1* in poplar and our results are consistent with those seen in other species.

Materials and Methods

Lignin-modification vectors

To alter lignin quantity and quality, we manipulated the expression of four genes in the lignin biosynthetic pathway: *C4H*, *C3'H*, *F5H*, and *COMT*. This combination allows us to evaluate the impact of decreased lignin content (*C4H*, *C3'H*), as well as changes in monomer composition (*C3'H*, *F5H*, *COMT*) on the ease with which cell walls can be deconstructed. Combined *F5H* up- and *COMT* down-regulation should result in plants with 5-hydroxyguaiacyl subunits. This type of lignin is not known to occur naturally, but could be of interest with respect bioenergy. Poplar cDNA clones were provided by Jörg Bohlmann (UBC). *Arabidopsis F5H* cDNA was previously isolated (Meyer et al. 1996). To generate entry clones, fragments were restricted from cDNA clones and sub-cloned into an entry vector. To generate RNAi constructs, entry clones were recombined with a destination vector using the Gateway LR cloning system. To generate over-expression vectors, entry clones were recombined with over-expression destination vector using LR. To drive expression of RNAi transcripts, we used both the CaMV *35S* and the *Arabidopsis C4H* promoters.

Corngrass1 vectors

A 0.6-Kb DNA fragment containing the *Cg1* transcript (Accession # EF541486) was cloned into the binary plasmid pK2GW7 (Karimi et al. 2002) by recombinase-mediated integration using LR clonase, positioning the *Cg1* transcript downstream from the 35S promoter and upstream of the *35S* terminator.

Producing transgenic plants

T-DNA from binary vectors was transformed into hybrid poplar clone INRA 717-1B4 (*Populus tremula* x *P. alba*) using an *Agrobacterium*-mediated transformation protocol (Meilan and Ma 2006). A minimum of 25 lines (independent transgenic events) was produced for each construct. Transformation of kanamycin-resistant rooted lines was verified via PCR; transgenic plants were acclimated in a greenhouse and shade frame before being transplanted outdoors (4 ramets/line). Material from field-grown trees was used to conduct analyses on lignin-modified plants; material from greenhouse-grown plants was used for analyses performed on the *Cg1* transgenics.

Lignin analyses

Lignin composition and content were determined using standard methods (i.e., Klason lignin, pyrolysis GC-MS, and DFRC analysis) that have been described previously (Franke et al. 2002).

Quantitative real-time PCR

Total RNA was extracted from the youngest fully unfurled leaf using the RNeasy Plant Mini Kit (Qiagen). Primers were designed using PrimerExpress, and reverse transcription were performed using the High-capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Reactions (3 technical replicates) were performed using Fast SYBR Green Master Mix (Applied Biosystems). Expression level was normalized to that of the alpha tubulin gene (*TUA2*).

Plant measurements

Three ramets (biological replicates) of *in vitro* regenerated WT and 35S:*Cg1* transgenic lines, all at 5 months of age, were measured for height, stem diameter (5 cm above soil level), number of branches, number of leaves, number of nodes, internode lengths, and rate of leaf initiation. Plastochron index was measured as described by Erickson and Michelini (1957).

Results and Discussion

Because of position effects, the RNAi vectors were effective to varying degrees in the lines. For each vector, the 8 lines with the lowest level of native gene expression were propagated for transfer to the field. Relative expression for the *COMT* RNAi lines is shown in Figure 1. Syringyl (S) content of lignin in WT poplar is ~60%; our best-performing *COMT* RNAi lines had an S content of <10%. Currently, we are using plant material from field-grown trees for pre-

treatment and hydrolytic experiments. In addition, we are collaborating with an entomologist and pathologist to evaluate the transgenics' susceptibility to pests.

When *Cg1* was over-expressed in poplar, plants exhibited significantly greater branching and leaf area, larger stipules, and shorter internodes, but the number of internodes remained unchanged (Figure 2). The increased leaf area was due to sylleptic branching, but the rate of leaf initiation was unaffected. The severity of the phenotype was positively correlated with *Cg1* expression level. In addition, transgenic lines had up to 30% less lignin and syringyl to guaiacyl ratio (S/G) was lower than in WT poplar or a control transgenic line having low *Cg1* expression. We are in the process of establishing a field study to compare biomass production of our *Cg1* and WT lines. It is yet to be determined whether MIR156 directly regulates lignin biosynthesis or if the observed lignin changes were indirect consequences of the developmental changes caused by *Cg1* over-expression. Nevertheless, plants expressing *Cg1* may have commercial value as a cellulosic feedstock for biofuel production and in the paper-manufacturing industry.

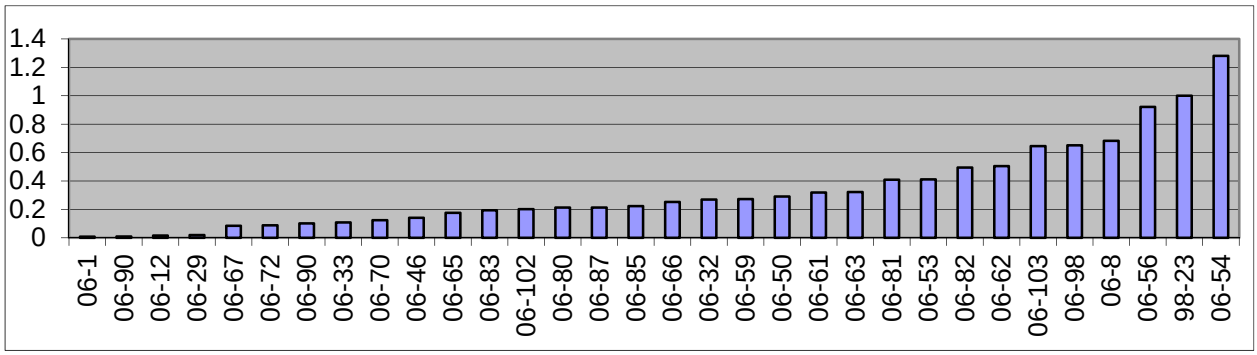

Figure 1. Relative expression of *COMT* (Y axis) for transgenic poplar lines (X axis) in which *COMT* was down-regulated using RNAi.

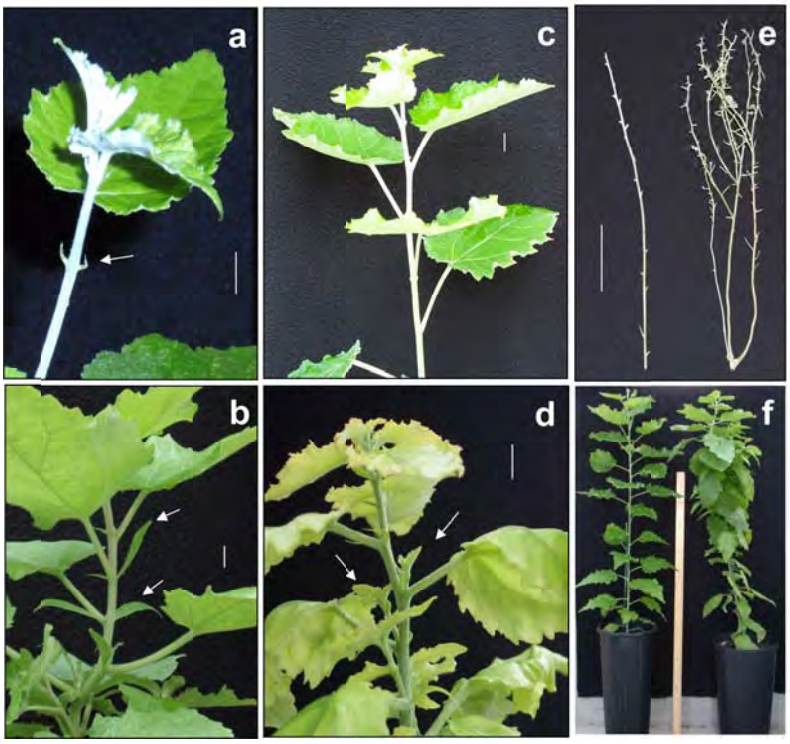

Figure 2. Comparison of wild-type (WT) and 35S:Cg1 poplar (INRA 717-1B4). A) Shoot apex of 5-month-old WT with stipule (arrow). B) Shoot apex of 5-month-old 35S:Cg1 with enlarged stipules (arrows). C) Shoot apex of 5-month-old WT. D) Shoot apex of 5-month-old 35S:Cg1 plant showing outgrowth of sylleptic branches (arrows). Scale bars in panels A, B, C, and D = 1 cm. E) An ~40-cm apical shoot from 1-year-old WT (left-hand side) and 35S:Cg1 (right-hand side) plants. Leaves were removed and petioles shortened to show branching. Scale bar = 10 cm. F) 7-month-old whole plants (WT on the left, 35S:Cg1 on the right). Ruler = 1 m.

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