# Field and pulping performances of transgenic trees with altered lignification

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The agronomic and pulping performance of transgenic trees with altered lignin has been evaluated in duplicated, long-term field trials. Poplars expressing cinnamyl alcohol dehydrogenase (CAD) or caffeate/5-hydroxyferulate *O*-methyltransferase (COMT) antisense transgenes were grown for four years at two sites, in France and England. The trees remained healthy throughout the trial. Growth indicators and interactions with insects were normal. No changes in soil microbial communities were detected beneath the transgenic trees. The expected modifications to lignin were maintained in the transgenics over four years, at both sites. Kraft pulping of tree trunks showed that the reduced-CAD lines had improved characteristics, allowing easier delignification, using smaller amounts of chemicals, while yielding more high-quality pulp. This work highlights the potential of engineering wood quality for more environmentally benign papermaking without interfering with tree growth or fitness.



Demand for paper is ever-increasing. At the dawn of a new millennium, the long-sought paperless society is far from being realized, with world pulp production in 2000 exceeding 185 million metric tons (~204 US short tons), of which nearly 70% was chemical (kraft and bisulfite) pulps. During chemical pulping, lignin is removed from cellulose and hemicellulose using costly and noxious chemicals. Economic and environmental benefits could result from the use of wood modified to facilitate kraft delignification. Lignin has therefore been a target for genetic engineering during the past decade[1,2]. Most of the genes involved in lignin biosynthesis have been manipulated in model species, and some experiments have been performed in trees such as poplar[3–8]. For example, suppression of cinnamyl alcohol dehydrogenase (CAD), the final enzyme in the biosynthesis of lignin monomers, results in lignin with altered structure[4,7]. Suppression of caffeate/5-hydroxyferulate *O*-methyltransferase (COMT), an enzyme involved in syringyl (S) lignin synthesis, results in dramatic reduction in S lignin content[3–5]. Transgenic experiments have significantly advanced our understanding of the lignin-biosynthetic pathway, and some of the poplars produced have shown preliminary promise for improved chemical pulping[4].

To date, only juvenile poplars grown in greenhouse conditions have been studied. For many reasons, it is essential to assess whether the characteristics observed in young greenhouse-grown plants are maintained in older trees under field conditions. Field-grown trees are subject to widely variable conditions that do not exist in the greenhouse (wind, rain, frost, etc.). Similarly, older field-grown wood may differ significantly from young greenhouse-produced wood in its response to kraft pulping. To be commercially viable, lignin-modified trees must show good agronomic performance and consistent pulping benefits, independent of growth environment. This can be assessed by coordinated trials at different sites.

Of equal importance to commercial considerations are the potential ecological impacts of growing transgenic lignin-modified trees on a large scale. Lignin, or its precursors, could influence the palatability (or otherwise) of leaves to herbivorous insects, the resistance of trees to pathogens, and the decomposition of plant residues by soil organisms. Such biological interaction can only be explored during monitored, regulated field releases of lignin-modified transgenics.

In this paper, we evaluate the agronomic and pulping performances of CAD- or COMT-deficient transgenic poplars grown in the field for four years in two different countries. The UK trial ended prematurely when vandalized by ecoactivists in July 1999, an act deemed an "own goal" by many commentators because of the potential environmental benefits of lignin-modified trees. Our results show that changes in lignin content or structure observed in greenhouse-grown trees[3,4,7] were maintained in older trees in both field trials. The lignin modifications had no unexpected biological or ecological impacts. Interactions with leaf-feeding insects, microbial pathogens, and soil organisms were unaltered, but the short-term rate of decomposition of transgenic roots was enhanced. To the best of our knowledge, this is the first duplicated field evaluation of transgenic trees and the first commercially relevant demonstration of the improved pulping performance of CAD-antisense poplars.

## Results

**Growth of field-grown transgenic poplars**. We evaluated five different lines: the nontransformed wild-type line (717-1-B4), two antisense lines deficient in COMT (ASOMT2B; ASOMT10B)[3], and two

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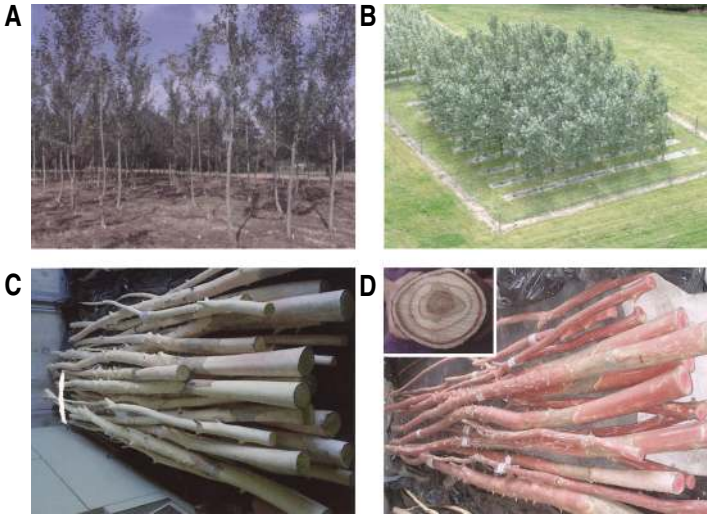


**Figure 1.** Field trials of transgenic poplars modified in lignin biosynthesis. (A) Field trial at Ardon, France. (B) Aerial photograph of field trial at Jealott's Hill, United Kingdom. (C) Debarked wild-type trunks harvested after 4 years' growth in the field. (D) Corresponding debarked ASCAD21 trunks. (Note: cut transverse ends of trunks have been marked with paint to identify the line; see inset for unpainted transverse section showing restriction of the red modified lignin color to developing xylem.)

antisense lines deficient in CAD (ASCAD21; ASCAD52)[7]. Field trials began in 1995 in Ardon, France and Jealott's Hill, United Kingdom (Fig. 1A, B). The sites differed in climate, soil conditions, and cultural practices. Tree phenology was recorded each spring. No difference in bud burst timing was evident for the transgenic lines, at either site, in any year (Fig. 2A). Growth parameters of all trees were measured annually. None of the transgenic lines showed any significant difference from wild-type trees in height or trunk diameter (Fig. 2B–E). In 1996, trees grown in France were on average taller than those in England, probably because of the milder climate. In 1997, trees of the French trial were cut down to induce vigorous stem regrowth without the need for staking. The new French stems remained smaller than the UK trees in subsequent years.

**Biological interactions**. Attacks by insects, fungi, and bacteria were continually monitored. No serious infestations occurred, and no differences were evident in the extent of damage between lines at either trial site. Leaf damage by caterpillars, sawflies, and leaf beetles, albeit minimal, was the most obvious biotic challenge to the trees. The leaf area lost through insect feeding was estimated each spring for the UK trial (Fig. 3A; Supplementary Fig. 1 online). Statistical analysis on the pooled data, from all years, for every damage category, showed no difference in the proportion of trees affected for any transgenic line compared to wild type (one-way analysis of variance (ANOVA) with angular transformation). Annual surveys showed that wild-type and transgenic trees harbored a similar variety of species, including caterpillars, shield bugs, froghoppers, ladybirds, ants, copper beetles, earwigs, and spiders. The most frequent pests were aphids and ants. Rust (*Melampsora* sp.) was occasionally observed, and transgenic and wild-type trees were equally affected.

**Enzyme activity and lignin structure**. At final harvest, CAD and COMT activities were measured in five randomly selected trees of each line (Fig. 4). Although activity was significantly higher than in younger greenhouse-grown trees[3], downregulation of COMT activity was still effective in ASOMT lines after four years' growth. ASOMT10B and ASOMT2B mean activities were, respectively, 32% and 44% of wild-type activity (Fig. 4A). CAD was substantially

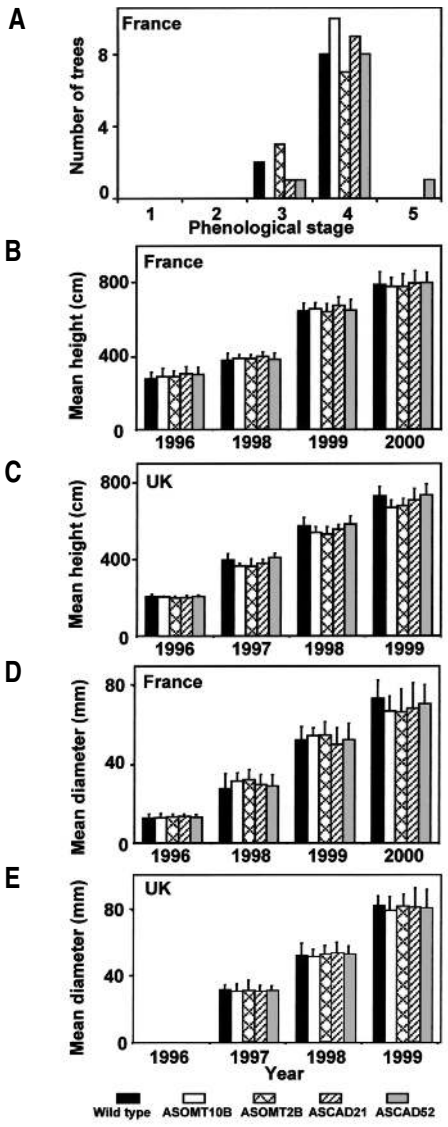


**Figure 2.** Agronomic traits of field-grown transgenic poplars modified in lignin biosynthesis. (A) Notations (as previously defined[14]) for phenology were recorded for all trees during bud burst. Data are shown for one time point in 1999 for Ardon. Data from other time points, other years, or from the UK trial were similar, showing no consistent differences between genotypes. (B, C) Average height of transgenic lines from 1996 to 2000 in Ardon and Jealott's Hill, respectively. (D, E) Tree average diameter measured at breast height at Ardon and Jealott's Hill, respectively. Error bars represent s.d. from mean. All trees were included in the analysis (i.e., $n = 10$ per line, France; $n = 12$ per line, UK).

depleted in ASCAD21 trees (15% of wild type) and reduced to a lesser extent in ASCAD52 trees (47% of wild type) (Fig. 4B). Tree-to-tree variability in the transgenic lines was generally less than in the wild type.

As previously observed[3,7], developing xylem in field-grown CAD- or COMT-downregulated trees was, respectively, red or pale rose in color, as a result of modifications in lignin. This was particularly noticeable for debarked ASCAD21 trunks (Fig. 1D). Line ASCAD52, with greater and more variable CAD activity, showed more tree-to-tree variability in trunk color.

Lignin content and structure were determined for trees of the French trial after 24 and 48 months, and for 6-month-old siblings grown outdoors in a polyethylene tunnel. Wild-type and ASOMT

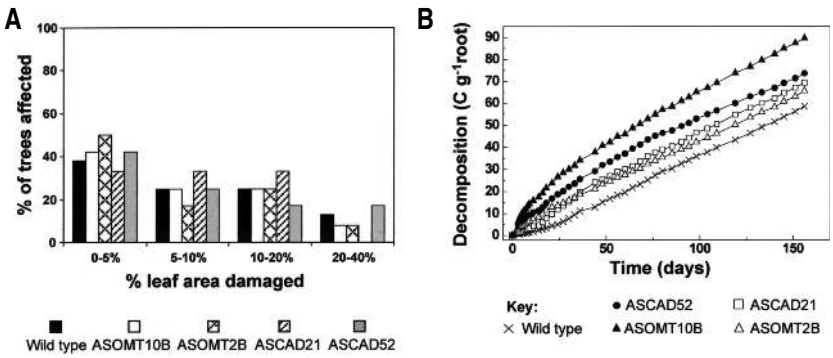


**Figure 3.** Biological interactions of lignin-modified transgenic poplars in the Jealott's Hill trial. (A) Leaf damage caused by feeding insects was recorded annually (data shown for 1997; see Supplementary Fig. 1 online for other years). (B) Decomposition of root material from transgenic and wild-type trees in a common test soil. Each point is the mean of three replicate analyses on root samples from three different trees per line. For clarity, error bars have been omitted, but s.d. was always <10% of the mean.

trees had similar Klason lignin contents when harvested at 48 months (Fig. 5A). Wood from the CAD-antisense poplars showed slightly reduced lignin content throughout development. This reduction was significant ($P < 0.05$) in ASCAD21, the line with least CAD activity. Lignin structure was evaluated by thioacidolysis, a technique that releases guaiacyl (G) and syringyl (S) monomers from lignin units only involved in β-O-4 bonds, providing an estimate of the frequency of these units. The proportion of lignin units only involved in β-O-4 bonds increased in all lines as the trees aged (Fig. 5B). However, transgenic lines, particularly ASOMTs, consistently had a lower frequency of such units, indicating a change in lignin structure. The relative proportion of S to G monomers (S/G) in thioacidolysis products was greatly reduced in ASOMT lines (Fig. 5C), and unusual 5-OH G units were evident (3 mol% for ASOMTs; trace amounts in other lines). CAD downregulation did not change S/G, but significant alteration to lignin structure was indicated by the increased frequency of free phenolic groups in ASCAD21 lignin, revealed by thioacidolysis of permethylated samples[4]. The frequency of free phenolic G units was 29.2 ± 1.5% for ASCAD21and 26.4 ± 0.6% for wild-type trees, while free phenolic S units, though scarce, were also increased (4.5% ASCAD21 vs. 3% wild type). These structural changes may facilitate kraft pulping by improving the solubility of lignin during the alkaline cook. Trees from the UK trial showed similar changes in lignin, albeit to a lower extent (Fig. 5A–C).

**Soil biology and root decomposition.** The trial's premature termination prevented comprehensive analysis of rhizosphere soil communities, planned for the UK trial. Nevertheless, a limited study was possible using soils collected from beneath the trees shortly after the emergency harvest. Soils were analyzed for C, N, microbial biomass, and microbial activity (basal respiration) (Supplementary Table 1 online). Interestingly, soil collected from a site under grass (<4 m adjacent) differed significantly from that under the trees, with more total C and N and greater microbial biomass and respiration ($P < 0.05$). There were no significant differences between soils beneath transgenic and wild-type trees for any of these parameters except basal respiration, which was reduced in two soils (ASCAD52, ASOMT2B; $P < 0.05$). This does not correlate with transgene expression (ASCAD52 and ASOMT2B are the lines with least modification to lignin) and may indicate spatial variability of soil properties in the field. Indeed, all of the respiration rates were at the lower end of the

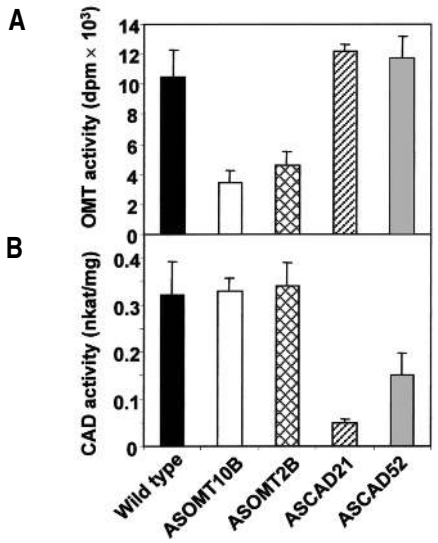


**Figure 4.** COMT and CAD activity in field-grown transgenic poplars. At final harvest (48 months; French trial), trees were assayed for (A) COMT and (B) CAD activity. The histogram shows the mean ± s.d., $n = 5$ for each line.

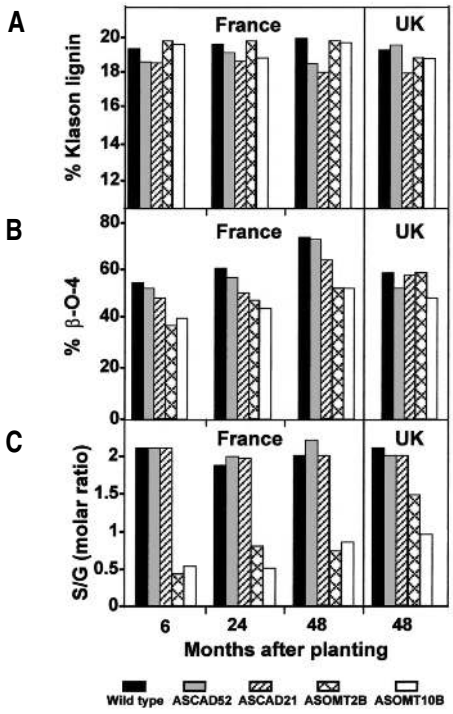


**Figure 5.** Lignin content and structure in field-grown transgenic poplars from both trials. Trees were harvested at 24 or 48 months post-planting. Six-month-old samples came from clonal siblings grown outdoors in a polyethylene tunnel. Pooled wood samples were prepared from chipped trunks of at least five trees per line. Each sample was subjected to duplicate or triplicate analyses. (A) Mean values for Klason lignin content of extractive-free stem xylem. Individual values varied from the mean by <1.5% (maximum s.d. = ±0.3). (B) Mean molar percentage of lignin units only involved in β-O-4 bonds (per 100 phenylpropane units[16]) in extractive-free xylem. Individual values varied by <3.5% of the mean. (C) Mean molar S/G ratios. Individual values varied from the mean by <3%.

normal range for temperate soils[9,10], probably because litter input from leaves was prevented by polyethylene sheeting, used to suppress weeds. The metabolic diversity in the soil microbial population was assessed on Biolog Ecoplates using 31 different C sources. There were no significant differences (assessed by one-way ANOVA) between soils for the substrates supporting microbial growth. Decomposition of root samples from the different genotypes in a test soil was monitored, via $CO_2$ production, over a five-month period (Fig. 3B). The transgenic lines produced more $CO_2$ than did the wild type, particularly over the first month of the experiment, indicating a greater extent of decomposition. One-way ANOVA showed that the enhanced decomposition was highly significant for ASOMT10B and ASCAD52 ($P < 0.01$) and significant for ASCAD21 ($P < 0.05$) and ASOMT2B ($P = 0.08$). Thioacidolysis and Klason analyses revealed that transgenic roots, like tree trunks, had normal amounts of lignin but altered lignin structure, with ASCAD21 and ASOMT10B showing the greatest modification.

**Table 1. Comparison of kraft pulping properties of wood from both field trials[a]**

| | 717 1B4 | | ASOMT10B | | ASCAD21 | |
|---|---|---|---|---|---|---|
| | France | UK | France | UK | France | UK |
| Kappa | 20.4 | 19.7 | 32.7 | 27.8 | 18.6 | 13.9 |
| Screened pulp yield (%) | 51.9 | 54.8 | 50.6 | 52.0 | 54.4 | 56.9 |
| Cellulose DP[b] | 2,090 | 2,190 | 2,030 | 2,100 | 2,140 | 2,270 |
| Brightness % ISO[c] | 89.8 | 89.3 | 89.4 | 88.5 | 90.1 | 90.7 |
| Breaking length, km ± s.d. | 8.57±0.02 | 9.24 | 9.05±0.04 | 9.26 | 9.05±0.04 | 9.90 |
| Tear index, $mNm^2/g$ ± s.d. | 6.41±0.38 | 4.28±0.21 | 6.19±0.26 | 6.36±0.33 | 5.90±0.40 | 5.12±0.31 |

[a]Kraft pulping was done at 18% active alkali charge. Results shown are the mean of duplicate analysis performed on a single wood sample (5–12 pooled, chipped tree trunks) per line. Standard deviations (predetermined for the method) are typically 1–3% of the mean.
[b]DP, degree of polymerization.
[c]Data presented on paper brightness, breaking length, and tear index are the mean results for 10 individual paper sheets made from each pulp and analyzed separately.

**Kraft pulping.** Kraft pulping was done under varying conditions to determine the optimum chemical charge for good delignification of wood from the different lines. Analyses were done initially on French trial wood with a sodium hydroxide charge range of 16–26%. Wood from ASCAD21, the line with lowest CAD activity, required a lower amount of chemicals than wild-type wood to reach the same kappa number, a parameter indicating the residual lignin content in pulp (Fig. 6A). For example, >17% active alkali was needed to produce pulp with a kappa number of 25 from wild-type wood, compared with 16% for ASCAD21 wood. This represents a 6% saving in the amount of alkali added. ASCOMT wood, however, needed more chemicals (~15% more active alkali) to be delignified to a kappa number of 25.

When the alkali charge was too low for efficient delignification (<18%; Fig. 6B), the screened pulp yield of wild type and ASOMT10B was poor, because of increased uncooked particles. For ASCAD21, the screened pulp yield was satisfactory even at low alkali charge. Therefore, this line can provide more pulp with fewer chemicals. To obtain a pulp with good mechanical properties, it is necessary during delignification to maintain cellulose quality, evaluated by its degree of polymerization (DP). For the same alkali charge, pulps of transgenic and wild-type lines showed a similar cellulose DP (Fig. 6C). As alkali charge increased, cellulose DP decreased. For a given kappa number, because less alkali was required to pulp ASCAD21 (Fig. 6A), cellulose DP was improved (Fig. 6D).

The kraft pulping properties of wood from both trial sites were compared at an alkali charge of 18% (Table 1). Wood from the UK trial exhibited slightly better pulping properties than wood from France. The improved performance of ASCAD21 in kraft cooking was reproduced but was not as marked as when using a lower alkali charge (Fig. 6A). For both trials, ASCAD21 wood could be pulped to a lower kappa number with higher yield while maintaining cellulose DP. After refining, the bleached pulps from CAD-deficient and wild-type samples had similarly good mechanical properties (breaking length/tear index) and brightness (Table 1).

The pulping experiments already described were done at 35% sulfidity. Subsequent independent experiments using different conditions and lower sulfidity (25%) yielded similar results, yet again confirming that pulp from line ASCAD21 had reduced kappa number and increased yield compared to wild-type pulp.

## Discussion

Our results demonstrate that the modified wood properties of CAD- and COMT-antisense trees were maintained over four years in the field at two different locations. The lignin contents and pulp kappa numbers for each genotype, not surprisingly, differed slightly between trial sites, but the overall trends were the same. Tree-to-tree variability in enzyme activity was less for the transgenic lines than for wild-type trees, indicating consistent levels of gene suppression. Similarly, changes in lignin content or structure in the transgenic lines were stable over time. Data from the field-grown trees were generally consistent with those previously obtained for greenhouse-grown plants, although for ASOMT lines, enzyme activity was higher and lignin changes less pronounced in the field material. No major interactions between genotype and environment were obvious; each line behaved similarly in both trials. The annual growth increment was lower for trees grown in the United Kingdom than for those grown in France, probably because of the less favorable cli-

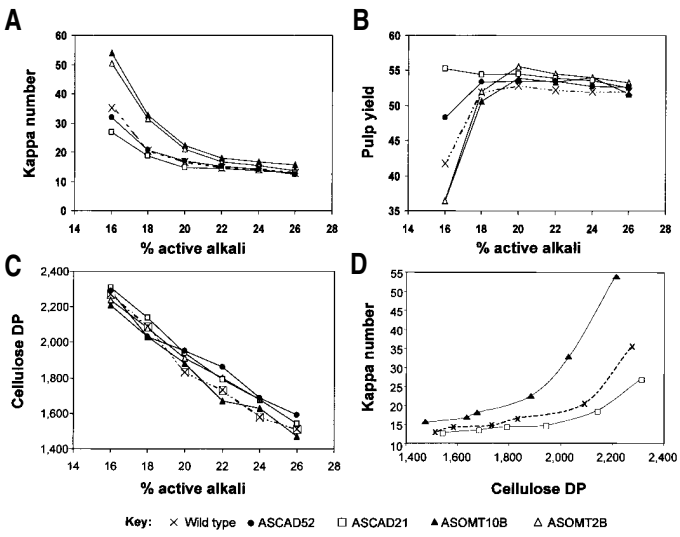


**Figure 6.** Kraft pulping characteristics of field-grown transgenic poplars. At final harvest (48 months; French trial), trunks from five trees per line were debarked, chipped, and pooled. Kraft pulping was done on these pooled samples at different alkali charges. (A) Kappa number. (B) Screened pulp yield. (C) Cellulose degree of polymerization (DP). (D) Selectivity curve: kappa versus cellulose DP. Results shown are the mean of duplicate analysis. s.d. (predetermined) is 1–3% of the mean.

mate. This may explain the lower lignin content and better pulping characteristics of the UK-grown trees.

Although CAD and COMT downregulation had no adverse effect on the field performance of the lines evaluated here, ongoing greenhouse tests suggest that growth can be affected in other lines with extremely severe CAD suppression (data not shown). Therefore, while specific changes can be made to lignin without affecting tree growth and development, our data highlight the need for rigorous field-testing during the selection of potentially useful genotypes. The transgenic lines selected for this study also behaved normally in biological interactions with other organisms. Similar profiles of visiting/resident insects were observed for transgenic and wild-type trees, and soil microbial diversity appeared to be unchanged. Only the soil taken from adjacent grassland had distinct characteristics. This highlights the normal, extensive ecological differences that exist between different types of vegetation grown within meters of each other on the same soil. Concerns about the ecological impacts of transgenic plants are therefore most sensibly considered against the scale of variability that already exists in nature. Indeed, plants with greater deficiencies in CAD and COMT activity than the poplars studied here arise naturally, by mutation, in plant populations[11,12].

One environmental process we predicted might be altered was the decomposition rate of plant residues in soil. This partly depends on lignin content, as lignin is inherently recalcitrant to decay and protects associated cell wall carbohydrates from microbial attack. A slight increase in decomposition rate was detected for roots of the field-grown transgenic trees. This was most obvious at early time points in the experiment, suggesting that the modified lignin may be less effective at protecting labile polysaccharide components from microbial and enzymatic attack[9].

Among the four transgenic lines evaluated, ASCAD21 holds real promise. It has good field performance and improved kraft pulping properties due to the slight decrease in lignin content and changes in lignin structure (increased frequency in free phenolic units) that enhance lignin solubility in alkaline medium. ASCAD21 is more easily delignified using smaller amounts of chemicals and yields more pulp with less cellulose degradation. Conversely, our data definitively establish that COMT downregulation leads to a lignin network that is less amenable to chemical pulping, although this material might still be interesting for other applications.

This study demonstrates the feasibility of producing, through genetic engineering, a wood more easily processed by kraft pulping, saving on energy and pollutant chemicals, and producing pulp with improved properties. Analysis of four-year-old trees has some limitations, but does translate to commercial situations. Short-rotation poplar is harvested after 6–12 years, and pulp properties stabilize after 2 years' growth. Changes in pulping character of the magnitude that we describe here (6% savings in chemical use and/or 2–3% increase in pulp yield in ASCAD21) are commercially valuable. However, it may well be possible to make even greater improvements in the future using the same genetic manipulation strategy. Our work highlights the need for long-term field trials of transgenic trees to evaluate fully the effects of any given genetic modification[13].

## Experimental protocol

**Trial design and monitoring**. Two lines (ASCAD21 and ASCAD52) were downregulated for CAD activity[7] and two (ASOMT2B and ASOMT10B) for COMT activity[3] using antisense transgenes in the INRA 717-1-B4 *Populus tremula* × *Populus alba* female clone. In Ardon (France), poplars were micropropagated, then acclimatized in the greenhouse in January 1995. In July 1995, upon evaluation by the Commission du Génie Biomoléculaire (file no. 95-03-05) and authorization (no. 95-43) from the Ministère de l'Agriculture, trees were planted in the field at 1.5 × 3 m density. Five repeats of two plants per line were surrounded by a border of wild-type trees to limit edge effects. A 3 m–wide safety zone surrounding the trial was kept clear of culture. Due to delay in planting authorization, trees had to be staked. The UK trial (approved by Department of Environment, Transport, and the Regions; Reference no. 95/R1/4) was set up at Syngenta's Jealott's Hill International Research Station in Berkshire. Conditions were similar to the French trial, although 12 trees per line were planted at 2 × 2 m and staked for the duration of the trial. Stem height (main stem) and diameter (at 1.2 m height) were measured annually. The timing of bud burst was scored for both trials, taking three series of measurements (4–7 days apart) during the critical period of bud burst in spring, using the phenology notations of the European Forest Genetic Resources Programme (EUFORGEN)[14]. Assessments of pathogen attack were done annually on one branch per tree of the UK trial, following a standard survey protocol from the Forestry Commission of Great Britain. Leaf damage was assigned to a damage class and incidence of insects, fungal lesions, disease, and frost damage recorded.

In March 1997, trees in Ardon were cut down (24-month samples) and stakes removed to encourage strong growth of new stems. Therefore, when samples were collected for analyses in June/July 1999, French stems were two years old grown from four-year-old rootstock, whereas UK trees were four years old (48-month samples). For each tree harvested (five trees per line, Ardon; 12 trees per line, United Kingdom), the main stem was cropped, debarked, and chipped for lignin analysis and pulping. A 5-cm-thick slice (taken at 2 m) was frozen in liquid nitrogen for enzyme activity determinations.

**Enzyme assay**. Assays were done on individual trees (five per line). CAD was measured in crude protein extracts as previously described[15]. COMT was assayed on the same extract in the presence of caffeic acid and tritiated *S*-adenosyl methionine[3].

**Lignin analysis**. Analyses were run on duplicate or triplicate samples of ground and extractive-free material prepared from pooled chipped wood of 5 trees per line (French trial) or 12 trees per line (UK trial) harvested 24 months post-planting (French trial) or 48 months post-planting (both trials). Siblings of the field-grown trees, kept in an outdoor polyethylene tunnel until six months old, were included in the analysis (five plants per line). Lignin content was determined by the Klason method[16]. Lignin structure was evaluated by thioacidolysis, before and after methylation[17]. Lignin-derived monomers were identified by GC-MS (Varian Saturn 2000, Varian, Walnut Creek, CA).

**Chemical pulp analysis**. Kraft pulping[18] was simulated at lab scale on 200 g oven-dried wood (same pooled samples as for lignin analysis) in small pressurized reactors in a rotating oil-thermostatic bath under the following conditions: active alkali = 16–26%, sulfidity = 35%, liquor/wood ratio = 4, temperature raised to 170°C over 90 min and maintained for 1 h. Pulps were washed and screened on a 150 µm sieve to determine uncooked particles and screened pulp yield. Kappa number and cellulose DP were determined according to international standards (NF ISO 302 and ISO 5351-1). Comparisons between both field trials for kraft pulping properties were done at 18% active alkali. Kraft pulps were bleached with the OD1E/OD2 sequence using the following four conditions: (i) oxygen stage (O): 3% NaOH, 5 bar $O_2$, 0.5% $MgSO_4 \cdot 7H_2O$, 90°C, 60 min, 10% consistency; (ii) chlorine dioxide stage (D1): 1.5% $ClO_2$, 70°C, 120 min, 10% consistency; (iii) extraction stage (E/O): 2% NaOH, 2 bar $O_2$, 70°C, 60 min, 10% consistency; (iv) chlorine dioxide stage (D2): 0.3% $ClO_2$, 70°C, 240 min, 10% consistency. Bleached pulps were refined in a PFI mill (3,000 revolutions) and pulp quality evaluated on 75 g/m handsheets (10 sheets per plant line) according to AFNOR or ISO standards. Fiber characteristics were measured with PQM 1000 (Metso Paper, Sundsvall, Sweden) and CyberMetrics (Atlanta, GA) analyzers.

**Soil characteristics and root decomposition.** At the UK site, soil samples were collected with an auger at a depth of 0–10 cm from beneath three trees per line in September 1999. For each tree, three soil samples were taken from different positions 10–30 cm from the trunks, and mixed to give one bulked sample per tree. Three soil samples were also collected from under grass in the field surrounding the trial. Samples of major roots were taken from beneath three trees per line. C and N determinations were made with a Carlo–Erba CHN analyzer (Carlo–Erba Strumentazione, Milan, Italy). Soil basal respiration and microbial biomass C were measured as described[10]. Microbial metabolic diversity was estimated from 5 g soil on Biolog Ecoplates (Biolog, Hayward, CA), monitoring utilization of 31 different C sources by the reduction of tetrazolium. Data were analyzed by one-way ANOVA.

Decomposition was estimated from $CO_2$ production of root (300 mg) samples buried in a sandy loam soil as described[9].

**Statistical analyses.** Mean values are quoted ± s.d. Unless otherwise stated, significance was tested at $P < 0.05$. ANOVA was done using Corel Quattro Pro statistical package (Corel, Ottawa, ON, Canada).

*Note: Supplementary information is available on the* Nature Biotechnology *website.*

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