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REVIEW

Ecological impacts of trees with modified lignin

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Abstract Few experiments have yet been performed to explore the potential ecological impacts of genetic modification in long-lifespan species such as trees. In this paper, we review the available data on GM trees with modified lignin focussing on the results of the first long-term field trials of such trees. These trials evaluated poplars expressing antisense transgenes to reduce the expression of the lignin biosynthesis genes cinnamyl alcohol dehydrogenase (CAD) or caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) with the aim of producing trees with improved pulping characteristics. The trees were grown for 4 years at two sites in France and England, and their ecological impacts and agronomic performance were assessed. Modifications to lignin in the poplars were maintained over the 4 years of the trial. The trees remained healthy throughout and growth was normal. The lignin modifications had no unexpected biological or ecological impacts. Interactions with leaf-feeding insects, microbial pathogens and soil organisms were unaltered although the short-term decomposition of transgenic roots was slightly enhanced. Investigation of the ecological impacts of the GM trees was curtailed by the early termination of the field

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trial when it was attacked and largely destroyed by anti-GM protestors. To supplement our work on the decomposition of GM plant materials with modified lignin, we have therefore turned to the study of transgenic tobacco lines where we can perform more comprehensive and controlled analyses of the biological and ecological effects of lignin-gene suppression.

Keywords Ecological impacts · GM trees · Lignin

Introduction

The public debate about the potential and risks of the widespread use of genetically modified (GM) plants has increased awareness and concern about how such plants might directly or indirectly impact on ecosystems or the environment. Experiments such as the recent farm-scale evaluations of GM crops in the UK, currently the largest scientific study of its kind anywhere in the world, have yielded significant amounts of information on the ecological impacts of herbicide-resistant annual crops such as maize, oilseed rape and sugar beet (Firbank 2003 and references therein). By comparison, hardly any experiments have been performed to explore the potential ecological effects of longer-lifespan species including GM trees. A range of useful traits have already been introduced into readily transformable forest tree species to manipulate characteristics such as biomass production, herbicide tolerance, disease resistance and fibre quality (Halpin and Boerjan 2003). A particular target for fibre improvement is to make lignin easier to separate from cellulose during pulping to allow more environmentally friendly processing, yielding both cleaner pulp and paper that is less prone to yellowing. Over the past decade, many research groups have produced plants with genetic modifications to lignin biosynthesis with a view to improving wood delignification during pulp and paper production, or to enhance forage digestibility (Baucher et al. 2003; Halpin 2004). Some of these modified-lignin plants have proven benefits for industry or agriculture and a small number of field trial evaluations have already taken place.

As the second most abundant biopolymer on Earth, lignin plays a critical role in the biogeochemical carbon cycle and affects the interaction between plants and other living organisms in a myriad of ways. Changes to lignin in genetically modified plants therefore have the potential to influence biological interactions with other organisms. Huge variability in lignin already exists between different tree and crop species, and in naturally occurring mutants. It is possible, indeed it is likely, that this natural variability already encompasses the kinds of lignin modification currently being introduced into pulp trees and other plant species by transgenic approaches (Monties 1998). The potential ecological effects of growing lignin-modified trees should be considered before their widespread deployment. Comparison of these effects, if they exist, with appropriate baseline studies of natural and agricultural plant populations, will enable evaluation of whether they are likely to have any real environmental or ecological impact.

The various ways modified lignin might influence biotic interactions both above-ground and below-ground is envisaged in Fig. 1. Aerial parts of plants provide shelter and food for a variety of visiting or resident birds, animals, insects and microbes. Whilst there is no reason to suppose that modified lignin would influence many of these interactions, some specific interactions deserve closer scrutiny. Modified lignin could alter a tree's defence against invading pathogens because lignin is often deposited during defence reactions in plants. Leaf feeding or stem boring insects might be able to detect and respond to physical and chemical changes in cell wall composition that might make plant materials more or less palatable and easier or more difficult to attack and digest. Large herbivores such as rabbits or deer might be able to inflict more, or less, damage to tree trunks. Below-ground, root exudates and dead plant material are major contributors to the organic matter input to soil and exert a range of effects on both microbial and faunal communities, on specific interactions between soil organisms, and on ecosystem processes such as decomposition and nutrient transformations. We have directly evaluated the influence of modified lignin trees on these biotic interactions in a long-term field trial study, evaluating as many above- and below-ground interactions as possible.

Evaluating transgenic poplars with modified lignin

Field trial design and sampling

Our trials were the first long-term field evaluations of GM trees with modified lignin and ran for 4 years between 1995 and 1999 at Jealott's Hill, Berkshire UK and at INRA Ardon, France. The trees were suppressed in expression of the lignin biosynthesis genes caffeic acid/5-hydroxyferulic acid *O*-methyl transferase (COMT) and cinnamyl alcohol dehydrogenase (CAD). The agronomic performance of the trees was assessed throughout growth at both sites whilst assessment of the ecological impacts of the trees was performed only on the UK trial. Small tissue culture plants, provided by the INRA group who had produced them, were acclimatised to soil and outdoor growth and planted in the field in June 1995. Trees were placed in a randomised block design and included two lines expressing a CAD antisense gene (ASCAD21 and ASCAD52, 12 trees each; Baucher

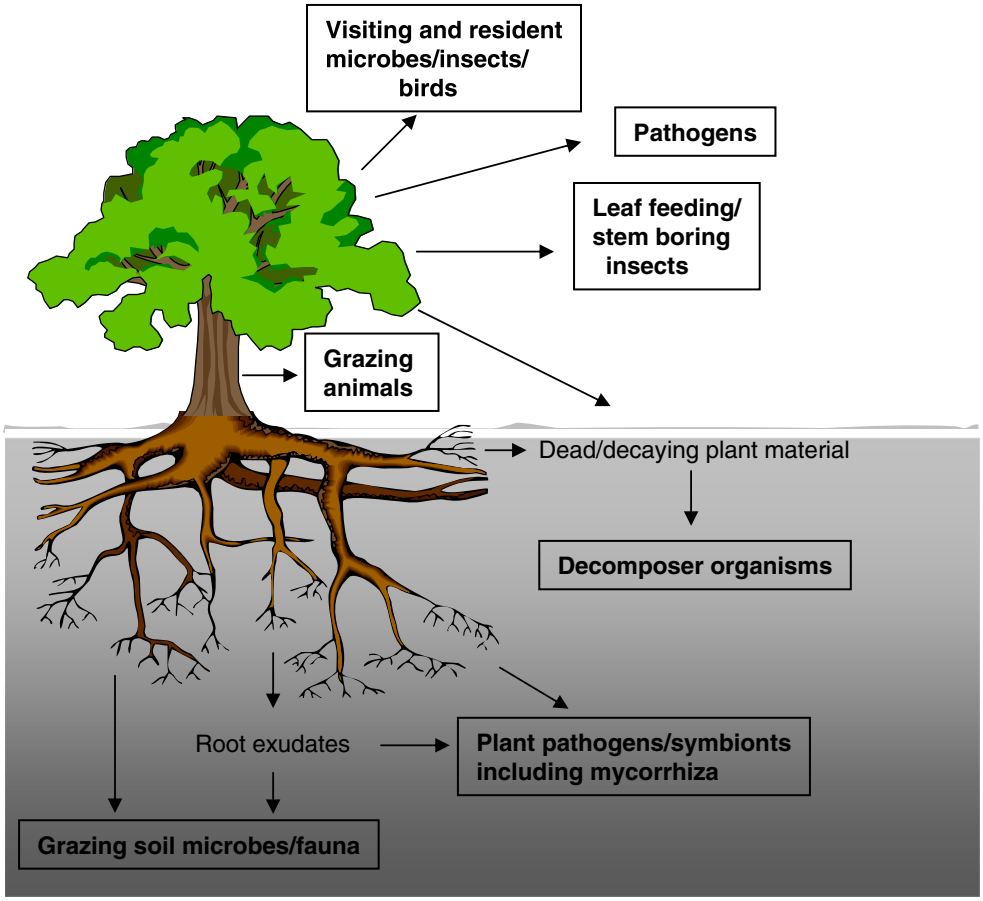

Fig. 1 Principal biotic interactions potentially influenced by lignin in the biosphere of a tree

et al. 1996), two lines expressing a COMT antisense gene (ASOMT2B and ASOMT10B, 12 trees each; Van Doorsselaere et al. 1995) and 24 wild type trees. There was no possibility of transgene escape as the trees were *Populus tremula*× *Populus alba* female clones that were harvested after 4 years, before poplars normally flower in the English and French climates. The trees were monitored regularly throughout the duration of the trial and no flowers were detected. Rabbits were excluded from the site by fencing, and tree protectors surrounded the trunks for up to 2 years post planting. Regular surveys recorded the incidence of resident and visiting insects on the trees and scored all visible damage and disease symptoms. The trees grew normally and there were no differences between the wild type and transgenic trees in height, trunk diameter or timing of bud burst in any year (Pilate et al. 2002). The UK trial was terminated prematurely after the trees were vandalised by anti-GM activists in July 1999. The trunks were harvested and subjected to pilot scale Kraft pulping to evaluate the potential benefits of the modified trees for papermaking. Small samples of trunk material were reserved for molecular, biochemical and chemical analysis of the degree of gene and enzyme suppression in the trees and for detailed lignin analysis. Samples of the trunk, roots and soil beneath the trees were taken for evaluation of the effect of the modified lignin on decomposition and on soil properties and biota. The early termination of the trial by anonymous anti-GM activists, reportedly on the grounds that they posed an environmental threat, prevented evaluation of certain below-ground ecological relationships of the trees, such as investigation of mycorrhizal associations, which would have required sampling of the trees whilst still alive.

Lignin modifications and pulping improvements in the transgenic poplars

Lignin content, composition and structure were evaluated in wood from poplar trunks from both trials (Pilate et al. 2002) and in root samples from the UK trial. Klason lignin analysis indicated that lignin content in the roots of the UK trees was higher than in the corresponding tree trunks, averaging 21% of the weight of extract-free xylem compared to an average of 19% for tree trunks. In contrast to trunks, where the ASCAD21 line had slightly reduced lignin content compared to wild type, the roots of all four transgenic lines, whether reduced in CAD or COMT, had similar levels of lignin to roots from wild type trees

(Table 1). The structure and composition of the polymer was analysed by thioacidolysis, a degradative technique that releases syringyl (S) and guaiacyl (G) units that are only involved in β-O-4 bonds within the polymer. Yields of thioacidolysis monomers were not greatly altered in trunks or roots of any genotype with the exception of ASOMT10B trunks where lignin units only involved in β-O-4 bonds decreased from 58 mol% in the wild type to 48 mol% in the transgenic trees (data not shown). The relative proportion of S to G monomers (S/G) was greatly reduced in both COMT-suppressed lines in both trunks and roots, consistent with the role of COMT in the production of S monomers. In conjunction with this change, novel 5-OH-G units, derived from the precursors used by COMT to make S lignin monomers, were recovered in the thioacidolysis products of trunk and root lignin from both COMT-antisense lines whereas they were almost undetectable in wild type lignin (Table 1). Although the S/G ratio was not altered in the trunks of the CAD-antisense lines, an effect of CAD suppression was more easily seen in the root lignin where both lines showed a reduction in S/G. In addition, 'marker' indene derivatives that have recently been demonstrated to be derived from the CAD substrate sinapaldehyde, incorporated into lignin by β-O-4 linkages (Kim et al. 2002), appear in the trunk and root lignin of the ASCAD21 line (Table 1). Thus, the characteristic lignin changes associated with CAD- or COMT-suppression that have already been well-documented for plant stems can also be detected in root lignin which appears to be modified in a similar way. Kraft pulping of wood from the tree trunks revealed that the reduced-CAD lines had improved characteristics, allowing easier delignification, using less chemicals, whilst yielding more high-quality pulp (Pilate et al. 2002). Pulping of the reduced-COMT lines, however, required more chemical to reach a similar level of delignification to the wild type plants (Pilate et al. 2002).

Assessment of potential above-ground ecological effects

Visiting and feeding insects

Whilst the trial was still growing, a survey of resident and visiting insects was performed in the summer of each year. All trees, both wild type and transgenic, harboured a wide variety of insects including ladybirds, ants, aphids, copper beetles, earwigs, shield bugs, and froghoppers as well as spiders. Caterpillars on the trees were putatively identified as those of Poplar Hawk Moth (*Laothoe populi*), Swallow prominent (*Pheosia tremula*), Yellow Tail Tussock (*Euproctis similis*), and Hornet Moth (*Sesia apiformis*). Insect infestation was recorded for one branch from each tree, leaf damage was scored on a 0 (no damage) to 5 (severe damage) scale and the percentage leaf area lost through damage was measured. Terminal shoots were checked for aphid infestation, galls and dieback. The most abundant pests were aphids and ants. Some aphids formed leaf nests but there was no evidence of true galls. Damage recorded was usually the result of caterpillar and larvae chewing on the leaves. Damage due to skeletonisers and leaf-mining insects was very minor. Overall, insect damage to the trees was very modest and both transgenic and wild type trees were equally affected (Pilate et al. 2002).

The effects that modified-lignin trees might have on leaf-feeding insects have recently been independently investigated by another research group under more controlled conditions. In that study, the trees were

Table 1 Target enzyme activity and lignin content and composition in trunks and roots of 4-year-old field grown poplars

Genotype	CAD activity[a] (%)	COMT activity[a] (%)	Klason lignin % EXR		S/G		Sinapaldehyde % of S		5-OH-G % of G	
	Trunk	Trunk	Trunk[a]	Roots	Trunk	Roots	Trunk	Roots	Trunk	Roots
Wild type	100	100	18.95	20.66	2.03	1.56	0.09	0.12	tr	tr
ASCAD21	16	116	17.69	20.62	1.94	1.22	1.50	2.10	tr	tr
ASCAD52	47	111	19.25	21.74	1.94	1.19	0.15	nd	tr	tr
ASOMT2B	106	44	18.54	21.01	1.39	1.09	nd	nd	2	2
ASOMT10B	103	32	18.49	21.07	0.88	0.78	nd	nd	3	2

All data represents the average of measurements from 3–5 independent trees per genotype. Individual values typically varied by less than 5% of the mean, except for enzyme activity where there was more variability (Pilate et al. 2002). Enzyme activity was calculated as percentage (%) of the average wild type value. Sinapaldehyde and 5-OH G units in thioacidolysis products are expressed as percentage (%) of conventional S and G units, respectively. For further details on field trial design and sampling, see Pilate et al. 2002.

EXR Extractive-free xylem, *S/G* ratio of conventional syringyl (S) monomers to guaiacyl (G) monomers in thioacidolysis products, *nd* not determined, *tr* trace amount

[a] Data taken from Pilate et al. 2002.

greenhouse-grown transgenic silver birch harbouring a COMT gene expressed from the 35S CaMV promoter. These plants apparently have co-suppressed COMT activity, as evidenced by the reduced S/G ratio of thioacidolysis lignin monomers. Leaf material from those trees was fed to five species of common leaf-consuming insect herbivores, representing both lepidopteran and coleopteran species. The overall relative growth rates of the herbivores did not differ whether they were fed transgenic or wild type leaves although one invertebrate herbivore (*Phyllobius* sp.) apparently consumed more leaf vein (as opposed to leaf lamina) tissue when fed on one particular transgenic line (Tiimonen et al. 2005). Four out of the five insect herbivores showed no preference for any particular genotype in food selection experiments. However, one (*Aethalura punctulata* larvae) preferred leaves of one transgenic genotype, but did not show the same preference for the second transgenic genotype modified in exactly the same way. This preference is therefore probably not associated with the lignin modification. Thus, as in the UK field trial, changes to lignin content and composition had little or no effect on leaf-feeding insects.

Symptoms of disease

During the UK trial, all trees remained healthy over the 4 year period, and no significant incidence of disease was observed. Trees were continually monitored for disease symptoms caused by fungi, bacteria or viruses. Occasional discolouration of the leaf top, usually associated with brightly coloured spores on the underside of the leaf, was observed. This was putatively diagnosed as rust, probably caused by *Melampsora* spp., and was observed with equally low frequency on all tree genotypes. Chlorosis and other types of leaf distortion due to pathogen attack were not observed on any tree. Thus, there was no evidence that the transgenic trees were any less resistant to diseases in the field than their wild type counterparts.

Assessment of potential below-ground ecological effects

Decomposition of poplar roots and trunks

After the termination of the trial after 4 years' growth, samples of trunk wood, roots and soil were taken for decomposition studies. Decomposition of chopped root material from the wild type and transgenic genotypes was monitored over a 5-month period in a test soil. All of the transgenic genotypes evolved slightly more CO_2 than the wild type, indicating a greater rate of decomposition. The effect was small but significant and was most marked over the first month of the study when it is expected that labile components such as polysaccharides, rather than the more recalcitrant lignin, would be attacked. Because the transgenic genotypes had no change to lignin content, these data indicate the influence that changes to lignin structure and composition can have on decomposition. From these results, we hypothesised that the modified lignin may be less effective than wild type lignin at protecting more labile cell wall and intracellular components from microbial and enzymatic attack. Comparable observations have been made and investigated in more detail with materials from tobacco plants decomposing in soils (Hopkins et al. 2001; Webster et al. 2005; see below).

A more extensive and prolonged decomposition study was set up using the more abundant trunk wood from the field trial harvest (Tilston et al. 2004). Small sections of poplar trunks were incubated in three different soils for a total of 18 months. Decomposition was monitored over the first 77 days by measuring CO_2 evolved and the final amount of decomposition after 552 days was determined by total mass loss. In two of the soils, CO_2 production over the first 77 days was lower for most of the transgenic lines compared to the wild type, whilst in the third soil it was higher. However, none of these differences was statistically significant and the variation between replicates of the same line was greater than the variation between lines (Tilston et al. 2004). Similarly, the total mass losses over 18 months ranged between 35 and 62% on the different soils but the differences between transgenic and wild type lines were not consistent or significant (Fig. 2). These data suggest that the environmental variability during growth in the field

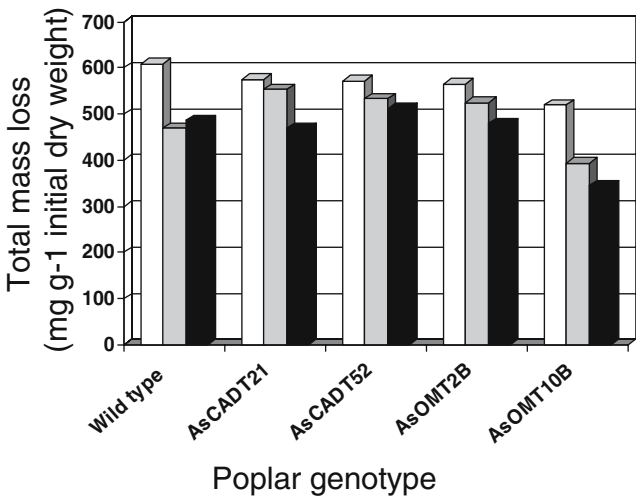

Fig. 2 Total mass loss from 4-year-old field grown poplar trunk material after 552 days of decomposition in soil. *Different colour bars* represent three different soils. *Each value* is the mean of three replicates. The average standard deviation from the mean between replicates was 17% but ranged from 4–45%. Drawn with data from Tilston et al. 2004

has had greater influence on subsequent wood decomposition than have the genetic modifications to lignin.

Soil properties and microbial biomass and composition beneath the poplars

Comprehensive analysis of the rhizosphere soil communities associated with the wild type and transgenic trees was prevented by the unexpected premature termination of the trial after it was attacked by anti-GM protesters. Nevertheless, at the time of the emergency harvest, enough soil was collected from beneath the trees to enable a limited study. This determined that there were no differences between soils taken from underneath transgenic trees and those taken from below wild type trees for C, N or microbial biomass (Pilate et al. 2002). Microbial activity, measured as basal respiration, was apparently reduced in soils under the transgenic lines ASCAD52 and ASOMT2B. This does not correlate consistently with transgene expression because basal respiration in soils from beneath ASCAD21 and ASOMT10B, the lines with greatest reductions in CAD and COMT, respectively (see Table 1), were no different from those of soils from beneath wild type trees. These differences may therefore be related to spatial variability of soil properties in the field. The soil microbial population was further analysed for its metabolic diversity on Biolog Ecoplates using 31 different C substrates. No significant changes in the substrates supporting microbial growth were evident for any of the soils from underneath transgenic trees compared to soil from wild type trees. It was notable that the most distinctive soil properties seen in this experiment were in soil samples taken from the site just outside the trial area (<4 m from the trees) which had been under grass during the lifetime of the field trial. These soils differed from the soil under the trees in all properties measured, having significantly more total C and N and higher microbial biomass and basal respiration (Pilate et al. 2002). Thus, on a single site, different types of vegetation can promote extensive ecological changes to the properties of the same soil. In our study, these influences of different vegetation types were far greater than the effects of genetic modification within a single species.

Microbial biomass was also determined at the end of the decomposition experiment where poplar trunk sections had been buried for 18 months in three different soils (Tilston et al. 2004). Whilst addition of trunk material increased the microbial biomass of all soils, there was no correlation between these increases and the total mass of trunk lost through decomposition over 552 days. Although some transgenic lines showed higher or lower soil microbial biomass compared to wild type for specific soils, no consistent trends existed. So, for example, COMT-suppression apparently led to a significant increase in soil microbial biomass when ASOMT2B trunks were buried in one soil but led to a decrease in soil microbial biomass when sections of the same trunks were buried in another soil. Thus, variations in soil conditions during the laboratory incubations were likely greater than any variation due to genetic modification.

Supplementary studies on decomposition and soil properties using transgenic tobacco

Decomposition of tobacco stems in soil

Access to field grown transgenic poplars was limited because of their relatively slow growth and the premature end of the field trials, so we complemented our poplar studies with a series of experiments on transgenic tobacco plants with similar modifications to lignin biosynthesis. This also allowed us to look at a wider variety of lignin modifications and to determine whether these modifications could potentially influence ecological interactions when plants are grown under optimum and controlled conditions as opposed to the more variable but natural conditions in the field.

Decomposition of stems from tobacco plants harbouring CAD and COMT antisense genes (Atanassova et al. 1995; Halpin et al. 1994) or a partial sense co-suppression transgene for cinnamoyl CoA-reductase (CCR) (O'Connell et al. 2002) was followed during a 77-day incubation in four different soils (Hopkins et al. 2001). Like the poplars with the same genetic modification, stems from CAD-antisense and COMT-antisense tobacco had similar lignin levels to wild type stems whilst CCR-suppressed plants had clearly reduced lignin content. Stem material from all of the transgenic lines decomposed significantly more rapidly than stem material from wild type plants in all of the soils with the exception of the CAD antisense stems in Stagnohumic gley soil where decomposition rates were unchanged compared to wild type. As with the poplar experiment, the differences in decomposition rate were most marked during the early stages of the experiment suggesting that labile components, rather than lignin, were being attacked The water contents of the plant lines differed, with the CAD- and COMT-antisense lines containing less water that wild type stems and the CCR-suppressed lines containing more water. These changes in water content could suggest altered porosity of the tissues, possibly due to changes in lignin hydrophobicity, which might be related to increased accessibility of the more labile plant components to microbial decomposition.

Further work (Webster et al. 2005) explored our hypothesis that the modified lignin in these transgenic tobacco lines led to increased decomposition rates because

it was less effective at protecting labile cell components from enzymatic attack. Stems from the transgenic and wild type plants were dissected into different fractions including (a) intact stems, (b) the insoluble stem fraction after water washing, (c) the lignin-rich woody xylem fraction obtained by removing the epidermis and cortex, and (d) the lignin-rich insoluble fraction obtained by washing the lignin-rich fraction with water. Decomposition experiments on the intact stems monitored over 74 days yielded identical results to our previous data, i.e. the total amount of CO_2 produced was significantly higher for CCR-suppressed stems than it was from other lines although CAD-antisense and COMT-antisense lines also produced significantly more CO_2 than wild type stems (Fig. 3). However, substantially increased total CO_2 production was only seen for the CCR-suppressed line when the lignin-rich fraction was assayed. Examining the kinetics of decay more closely revealed that both intact stems and the lignin-rich fractions of all lines showed a rapid initial flush of CO_2 production that peaked at 2–4 days and subsided by day 20 (Webster et al. 2005). These initial flushes of CO_2 were already significantly greater ($P<0.05$) for the transgenic lines than for the wild type. There was no initial flush from the insoluble fractions suggesting that the C that fuelled this decomposition activity was in the water-soluble component which subsequent analysis showed was dominated by soluble sugars. Moreover, the timing of the initial flush could be mimicked by adding glucose or alanine to soil in the absence of plant material, again suggesting that low molecular weight compounds in intact stem tissues, such as simple sugars and amino acids, might supply the C for the initial

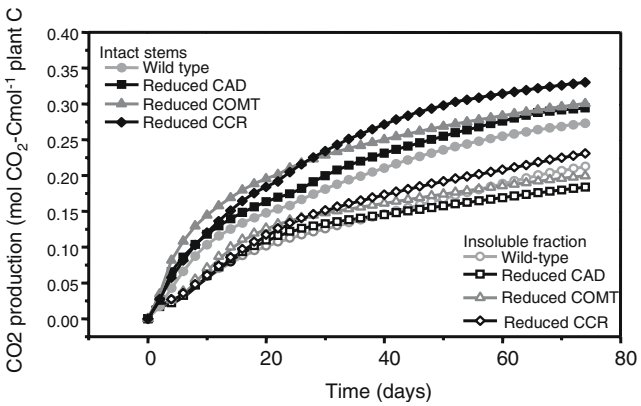

Fig. 3 Decomposition of different fractions from tobacco stems. Stems from transgenic and wild type plants were dissected into different fractions including intact stems and the insoluble stem fraction after water washing, and other fractions (see text). Decomposition was monitored over 74 days. The total amount of CO_2 produced was significantly higher for the intact stems of the transgenic lines compared to the wild type but these differences were largely lost when the insoluble stem fractions were analysed. *Each point* is the mean of experiments with three replicate plants. Standard deviations were typically less than 10% of the means, except during the first 10 days of the experiment

decomposition flush (Webster et al. 2005). Thus, it appears that the increase in decomposition rate compared to wild type in intact CAD-antisense and COMT-antisense stems results from the decomposition of water-labile fractions in the outer cortical and epidermal tissues, whereas in CCR-suppressed plants, additional characteristics of the lignin-rich fraction, most likely the reduced lignin content of the xylem, also contribute to the increase in decomposition. Subsequent work reinforced the distinction between the decomposition characteristics of the reduced-CCR plants and the other transgenic lines. When N was added (as alanine or NH_4NO_3) to the soil before the plant materials, the rate of subsequent decomposition was significantly increased for the reduced-CCR line but not for the other transgenic lines (Hopkins et al. 2006). This suggests that, for the reduced-CCR line only, the decomposition rate is constrained by the ready availability of N to decomposer organisms.

Microbial biomass and composition during decomposition of tobacco stems

To investigate short-term changes in soil microbial community structure when tobacco stem residues with reduced CAD and COMT decomposed in soil, phospholipid fatty acid (PLFA) profiles were determined over the first 14 days of decomposition, when the largest changes in decomposition rate occurred. The size of the soil microbial community did not differ between genotypes and, for most biomarkers, there were no significant changes in the relative abundances of individual PLFAs over time or between the different plant genotypes (Hénault et al. 2006). However, the fungal biomarker 18:2ω6c (linoleic acid) was the exception. It increased significantly with time in all of the soils to which plant material had been added but increased more rapidly for the transgenic genotypes compared to the wild type. It was also significantly more abundant at the end of the experiment for the reduced CAD material (which was also the fastest decomposing plant material) than for wild type or reduced-COMT lines. These data suggest that fungal colonisation of the modified plant materials occurred more rapidly than it did for wild type. Bearing in mind our earlier work indicating that the more rapid decomposition rates in the transgenic lines are related to greater availability of labile substrates, these new data may indicate that fungi make a substantial contribution to the utilisation of the labile fraction of the plant material.

Decomposition of tobacco roots in soil

The decomposition of roots of different lignin-modified tobacco genotypes was also assessed. Results showed that, unlike CAD-, CCR- or COMT-suppressed tobacco stems

where modifications to lignin consistently increase the rate of decomposition, the roots either decomposed more slowly or at the same rate as unmodified roots (Thain et al., unpublished data). Even roots of lines with reduced lignin decomposed to the same extent as wild type roots over a 42-day period. These results highlight the influence that the composition and architecture of different tissues (stems vs roots) can have on decomposition and indicate that, in tobacco roots, reduced lignin content does not necessarily result in enhanced decomposition.

This recent work also highlighted the major influences that experimental design can have on the results of decomposition experiments. For example, the growth history of the plants greatly influenced subsequent decomposition and roots from plants raised in tissue culture decomposed significantly faster than those from plants with the same genetic modification that had been raised from seed (Tilston et al., unpublished data). We attribute this result to an increase in soluble carbohydrate content in the roots of tissue culture propagated plants which persisted even after the plants were transplanted into soil/peat-based medium.

Similarly, the treatment of the plant material before its addition to the decomposition experiment can dramatically influence results. Fresh root material from COMT-antisense tobacco plants decomposed more readily than roots from wild type plants (Hopkins et al. 2005) whereas dried root material from the same lines did not show this enhanced decomposition. Most interestingly, when earthworms were added to these decomposition experiments, the differences in C and N mineralization between the fresh COMT-reduced roots and wild type roots were greatly reduced (Fig. 4) (Hopkins et al. 2005). This result is probably due to earthworm activity in incorporating plant litter into the soil by physically and biochemically conditioning it, thereby facilitating CO_2 production. This result therefore fits with our earlier hypothesis that increased decomposition resulting from CAD or COMT suppression is related to a change in the physical accessibility of the labile carbohydrates to microbial enzymatic attack. In wider ecological terms, this important observation of a diversity–function relationship involving soil microorganisms and key ecosystem processes (carbon and nitrogen mineralization) may indicate that the effect of the modifications to lignin biosynthesis on plant residue decomposition can be offset by diversity of the soil decomposer community.

The effect of modifications to lignin on the size, activity and composition of the rhizosphere community was also investigated. Redundancy analysis showed that the influence of genetic modifications to lignin biosynthesis on rhizosphere phospholipid fatty acid (PLFA) profiles was less significant than underlying physico-chemical relationships. A significant proportion (78.2%) of the diversity of rhizosphere PLFAs correlated most strongly with parameters associated with tissue-culture propagation, i.e. soluble carbohydrate concentration and root decomposability. An insignificant percentage (2.8%) of the PLFA diversity was ascribed to genetic modification.

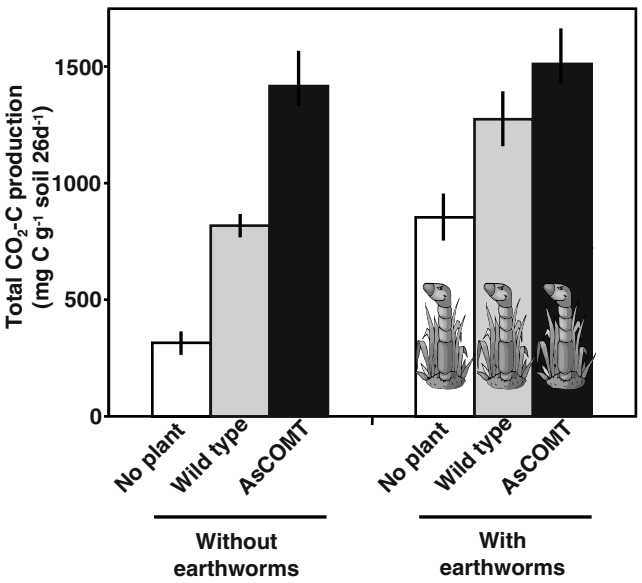

Fig. 4 Decomposition of tobacco roots in the presence of earthworms. Decomposition was monitored as total CO_2 produced when fresh tobacco roots were buried in soil, with and without earthworms, for 26 days. Drawn with data from Hopkins et al. 2005

Decomposition of tobacco roots by lignin by ligninolytic and cellulolytic fungi

Decomposition of the modified-lignin roots by specific microorganisms was investigated using isolates of *Chaetomium globosum* or *Phanerochaete chrysosporium*, which have cellulolytic and ligninolytic capabilities, respectively. There was a significant positive interaction on decomposition between *P. chrysosporium* and *C. globosum*. When root materials were colonised initially by *P. chrysosporium*, subsequent decomposition rates when the root residues were inoculated with *C. globosum* were significantly increased, but this was not seen when the fungi were inoculated in the reverse order (*C. globosum* inoculated before *P. chrysosporium*) (Tilston, unpublished data). We attribute this result to *P. chrysosporium* eroding the physical protection that lignin confers on labile cell wall components and allowing subsequent enhanced access to these labile components to *C. globosum*. All genotypes tested showed the same response to fungi and no significant differences were detected between the decomposition of the transgenic genotypes (CAD-suppressed, COMT-suppressed and a line suppressed in both CAD and COMT activities) and wild type plants.

Influence of tobacco root exudates on soil microbes

We rationalised that the changes to phenylpropanoid metabolism in our lignin-modified tobacco plants could potentially alter the composition of root exudates. Attempts to analyse the composition of exudates from the tobacco plants with modified lignin were unsuccessful due to the small amounts of exudate that could be collected. Evidence that the tobacco roots were exuding compounds into the rhizosphere and that these had a significant influence on soil microbes was provided by the following experiment. A decomposition assay was set up where root material was incubated in either the soil that the plants had been grown in or in 'pristine' soil that had not previously supported plant growth. Compared to the pristine soil, significantly less CO_2 was produced from the soil in which plants had been grown and there was a significant decrease in its microbial biomass after the addition of root material. This strongly suggests that the microbial communities in the soil where plants had grown were adapted to growth on carbon sources other than decaying root material. The prime candidate for an alternative carbon source in this soil is obviously root exudates. Thus, it seems likely that the tobacco lines we have been studying do, indeed, exude significant amounts of C into the rhizosphere and that this exudation significantly affects the soil microbial community. Further work is needed to explore this more fully.

Conclusions

As predicted, trees with modified lignin can exert slightly altered ecological influences compared to wild type trees but the effects are very small. No changes to relationships with visiting or leaf feeding insects or in disease susceptibility were identified during 4 years growth of CAD- and COMT-antisense poplars in the field. However, roots from the transgenic genotypes were found to decay slightly faster than wild type roots during the first month of decomposition, although the rate of decomposition of trunk material was not altered. Despite the increase in rate of decay of the root material, no consequent effects were detected on the biomass or diversity of the microbial population in the soil beneath the trees. Supplementary studies on similarly genetically modified tobacco plants confirmed that the size and diversity of the soil microbial community was not altered by the transgenic genotypes compared to wild type plants. However, fungal colonisation of the modified plant materials occurred more rapidly than it did for wild type, consistent with the more rapid initial decay kinetics for these genotypes.

In general, potential ecological impacts of modified-lignin plants were easier to detect using the tobacco lines which had been grown in controlled and optimised greenhouse conditions. Changes to decomposition rate were more obvious in these lines than they were in similarly modified but field-grown poplars that had been grown for a longer time. This suggests that the fluctuating nature of growth conditions in the field and over time, and the responses of plants to that changing and non-optimal environment, introduce more variability into the ecological relationships of the trees with soil microbes and decomposer organisms than does the genetic modifications to lignin. This is illustrated by the data on the decomposition of poplar trunk material where the variation between replicate trees of the same genotype was greater than the variation between genotypes (Tilston et al. 2004). Moreover, even when using tobacco grown in controlled conditions, the addition of earthworms to the assay system significantly reduced differences in decomposition between modified-lignin and wild-type tobacco plants (Hopkins et al. 2005). These results suggest that, in wider ecological terms, the effects of modifications to lignin biosynthesis on plant residue decomposition may be offset by diversity of the soil decomposer community. Thus, as assay systems move closer to 'natural' conditions, the influences exerted by genetic modifications to lignin are lost in the greater complexity and heterogeneity of the interactions that the more 'natural' conditions allow.

Although we have detected differential effects of transgenic plants with modified lignin during decomposition assays in vitro, the real issue in terms of risk assessment is whether such changes are likely to have any significant and lasting detrimental effect if similarly modified plants are widely grown in natural environments. Concerns about the potential ecological impacts of transgenic plants can only be sensibly considered within the context of the scale and impact of the variability that already exists in nature. Our analyses on field grown poplars indicated that soil properties and soil microbial biomass were not influenced by the lignin-modified trees but that the vegetation type (trees vs grass) had a major influence on all soil characteristics measured. In very controlled assay conditions using transgenic tobacco, we have been able to detect differences in decomposition rates between stems and roots of the same tobacco plants, between plants of the same genotype grown from seed or in tissue culture, and between similar samples used fresh or after air drying. These differences were of far greater magnitude than those recorded between equivalently treated samples from transgenic genotypes and wild type plants. In the natural environment, therefore, the diversity of plant litter encompassing different species and different parts of plants in various states of hydration and decay is likely to have greater influences on soil properties and ecological relationships with other organisms that the types of genetic

modifications to lignin studied here. Indeed non-transgenic plants with deficiencies in CAD and COMT and with similar modifications to lignin to some of the transgenics studied here are already grown in the USA and elsewhere. These naturally occurring mutants in maize, sorghum and loblolly pine are grown for their superior digestibility or pulping characteristics and have not been associated with any adverse ecological impacts. However, both mutant and transgenic plants with modified lignin offer a useful model system for further probing specific interactions between plant materials and decomposer and non-decomposer organisms in the soil environment.

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