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Assessment of *Populus* wood chemistry following the introduction of a Bt toxin gene

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Summary Unintended changes in plant physiology, anatomy and metabolism as a result of genetic engineering are a concern as more transgenic plants are commercially deployed in the ecosystem. We compared the cell wall chemical composition of three *Populus* lines (*Populus trichocarpa* Torr. & A. Gray × *Populus deltoides* Bartr. ex Marsh., *Populus trichocarpa* × *Populus nigra* L. and *Populus deltoides* × *Populus nigra*) genetically modified to express the Cry3A or Cry3B2 protein of *Bacillus thuringiensis* (Bt) with the cell wall chemistry of non-transformed isogenic control lines. Three genetically modified clones, each represented by 10 independent transgenic lines, were analyzed by pyrolysis molecular beam mass spectrometry, gas chromatography/mass spectrometry and traditional wet chemical analytical methods to assess changes in cell wall composition. Based on the outcome of these techniques, there were no comprehensive differences in chemical composition between the transgenic and control lines for any of the studied clones.

Keywords: Bacillus thuringiensis, cell wall chemical composition, cottonwood, metabolic profiling, transgenes.

Introduction

Populus species and hybrids have been advocated as model trees because of their small genomes, broad ecological and commercial importance and the ease with which they are genetically transformed (Tuskan et al. 2004). In commercial settings, insect damage is a major impediment to *Populus* plantation productivity and viability in many regions of the USA (Ostry et al. 1989, Fang et al. 2002). The cottonwood leaf beetle (CLB, *Chrysomela scripta)* is the principal insect pest in North American poplar plantations. Economic losses are primarily the result of defoliation caused by the larval stages of the CLB (Fang et al. 2002). Because CLB can produce up to seven generations per year (Earl Perkins, Perkins Specialty Spraying and Consulting, Inc. Hermiston, OR, personal communication), larval feeding can substantially reduce plantation biomass yields (Coyle et al. 2002).

Screening *Populus* clones for pest resistance is an integral part of most *Populus* breeding programs (Tuskan 1998). Although resistant genotypes have been found for some pests such as leaf rust (e.g., *Melampsora medusae*) and carpenterworm (*Prionoxystus robiniae*), CLB-resistant genotypes remain elusive. Currently, foliar application of pesticides, rather than deployment of resistant genotypes, is the primary means of insect control. Formulations containing acetyl-cholinesterase inhibitors are frequently the insecticide of choice for *Populus* plantations. These formulations provide effective control but can negatively impact beneficial insect populations (James 1997).

Bacillus thuringiensis (Bt) toxins are relatively selective insecticides that have few non-target effects (reviewed by James 1997). Commercial preparations of Bt toxins are frequently sprayed aerially to target larval stages. Specifically, the Cry3A toxin from Bt is highly toxic to the CLB when applied topically or expressed in transgenic plants (James et al. 1998). Therefore, transgenic plants could be an important CLB control option in hybrid poplar plantations, possibly lowering production costs while generating a targeted method of insect control. Field trials of transgenic poplars containing Bt transgenes are underway in several areas (e.g., Ellis and Raffa 1997, James et al. 1998, Huang and Wang 2003), most notably in China, France and the Pacific Northwestern USA (Meilan et al. 2000).

There is concern that genetically modified plants could have indirect or unanticipated adverse effects on the ecosystem (Noteborn et al. 2000, Raffa 2004). In support of this hypothesis, a recent study indicated that corn, genetically engineered with the *Cry1Ab* Bt gene, had a higher lignin content than the genotype from which it was derived (Saxena and Stotzky 2001). Lignin content in all of the genetically modified hybrids was 33–97% higher than in the non-transgenic isolines. Increased lignin content or changes in lignin structure as a re-

sult of genetic modification could have a detrimental effect on natural forests where microbial degradation of abscised plant materials (i.e., leaves, roots, limbs, etc.) is an important aspect of nutrient cycling (Hobbie 2000, Sariyildiz 2003). An increase in lignin would also negatively impact the economics and efficiency of processes that are dependent on delignification, such as papermaking and fermentation to produce ethanol (Dinus et al. 2001). Hardwood lignins have been shown to degrade and to have a faster rate of delignification than softwood lignins, which are composed predominately of cross-linked guaiacyl monomers (Chiang et al. 1988). Hence, it is important to characterize any transgenic plant materials before they are commercially deployed.

The analysis of biomass pyrolysis vapors by molecular beam mass spectrometry (pyMBMS) provides a method for rapid characterization of biomass samples. Although PyMBMS spectra for biological systems are generally highly complex and are often difficult to interpret, it has been shown that pyMBMS can provide detailed information about the cell wall chemistry of trees and other herbaceous biomass (Meuzelaar et al. 1982, Agblevor et al. 1994*a*, 1994*b*, Tuskan et al. 1999, Kelley et al. 2002). The technique is sensitive to changes in chemical composition of the cell wall and the peak intensities of the chemical compounds released during pyrolysis have been used to detect quantitative trait loci in loblolly pine (Sewell et al. 2002). In this study, we use a combination of pyMBMS and multivariate statistical analysis, along with gas chromatography/mass spectrometry and traditional wet chemistry, to characterize changes in the cell wall chemistry of *Populus* samples from trees grown under controlled field conditions after introduction of Bt toxin genes.

Materials and methods

Transgenic materials

Three hybrid poplar genotypes (Clone 50-197, *Populus trichocarpa* Torr. & A. Gray × *P. deltoides* Bartr. ex Marsh.; Clone 311-93, *P. trichocarpa* × *P. nigra* L.; and Clone OP-367: *P. deltoides* × *P. nigra*) were transformed with various Bt gene-containing constructs. Cottonwood transformation and regeneration were performed as described by Han et al. (2000). Briefly, sterile leaf discs were co-cultivated with *Agrobacterium tumefaciens* harboring the appropriate binary vector and shoots derived through organogenesis were cultured on a selective medium containing kanamycin and subsequently transferred to a root induction medium, where they were hardened off and placed in soil as rooted plantlets.

Clone 50-197 was transformed with two constructs (both provided by Monsanto): one with a *Cry3A* gene under the control of the promoter from an *Arabidopsis* gene encoding the small subunit of Rubisco; the other with a *Cry3B2* gene under the control of a constitutive figwort mosaic virus (FMV) promoter. Five independent transgenic lines were produced with each construct.

Two other constructs (also provided by Monsanto) were used to transform Clone 311-93. The first contained a *Cry3A* gene under the control of the CaMV 35S promoter; the other contained the *Cry3B2* gene under the control of the FMV promoter. Again, five independent transgenic lines were produced with each construct. All Monsanto constructs utilize the kanamycin resistance selectable marker gene (*NPT II*).

For Clone OP-367, nine independent transgenic lines were produced using the binary vector pKH20SBT-9, which contains two transcriptional units within its T-DNA. A map of the binary vector pKH20SBT-9 is shown in Figure 1. At the right-hand border, the *Cry3A* gene (supplied by Mycogen) is expressed under the control of the 35S promoter and the orf 25 terminator (Barker et al. 1983). Nearest the left-hand border is the *NPT II* gene under the control of the nopaline synthase (NOS) promoter and terminator. Matrix attachment region (MAR) elements (provided by S. Spiker, North Carolina State University) were positioned between the left-hand border and the NOS promoter and the right-hand border and the orf 25 terminator.

There were three types of non-transgenic controls for Clones 50-197 and 311-93: (1) ramets that had been propagated in vitro; (2) ramets that were propagated ex vitro in a greenhouse at Ohio State University; and (3) ramets that were propagated ex vitro by a commercial nursery. For Clone OP-367, there were two non-transgenic controls: (1) a line that had been regenerated in vitro (Line 85) and subsequently established in the field at the test site; and (2) a control that had never been in tissue culture (Line N). Cuttings for non-transgenic Line 85 were taken from trees grown on the east side of the Cascade Mountains (a xeric environment); cuttings for Line N were taken from stool beds grown at a commercial nursery in the Willamette Valley west of the Cascades (a maritime climate). All experimental plants produced for Clones 50-197 and 311-93 were established in the field using rooted plantlets. All OP-367 plants were established from dormant hardwood cuttings gathered during the winter and placed in cold storage until use. The field test site was located in southeastern WA (46°08′ N, 118°91′ W).

Wood samples were extracted with a 12-mm (diameter) increment borer. At the time of sampling, all ramets for Clones 50-197 and 311-93 were 1 year old; Clone OP-367 ramets were 3 years old. Increment cores were taken parallel to the direction of the prevailing wind (from the SSW) and through the entire tree. Branches and reaction wood were avoided during sampling. All cores were stored in 10-ml polypropylene, screw-cap-sealed, conical centrifuge tubes at 0 °C until samples were prepared for analysis.

Pyrolysis molecular beam mass spectroscopy

For Clones 50-197 and 311-93, one ramet of each transgenic line and one ramet of each of the three non-transgenic control lines were analyzed as biological replicates. For clone OP-367, biological replicates were analyzed from three ramets of each of the nine transgenic lines and each of the two non-transgenic control lines.

Oven-dried samples were ground to pass a 20-mesh sieve, weighed in quartz boats (~20 mg dry mass) and pyrolyzed in a reactor consisting of a quartz tube (2.5-cm inside diameter)

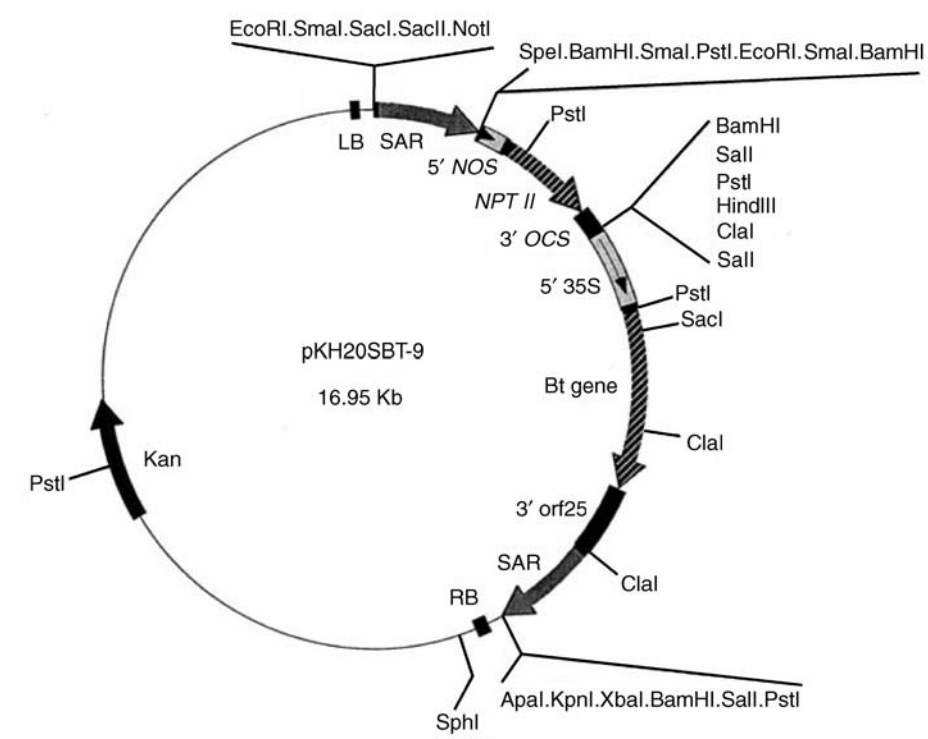

Figure 1. Map of the binary vector pKH20SBT-9. The *NPT II* gene is driven by the nopaline synthase (*NOS*) promoter and terminated by the octopine synthase (*OCS*) 3′ untranslated region. The Bt gene (*Cry3A*) was expressed under the control of the 35S promoter and the orf25 terminator. The transcriptional units are flanked by matrix attachment region (MAR) elements, which are just inside the left- and right-hand borders

with helium flowing through at 5 l min^{-1} (at STP). Three replicate subsamples (i.e., technical replicates) of each sample were analyzed. The reactor tube was placed such that the sampling orifice of the MBMS was inside the end of the quartz reactor. A custom-built MBMS based on an Extrel Model TQMS C50 mass spectrometer was used for pyrolysis vapor analysis (Evans and Milne 1987, Tuskan et al. 1999). The reactor was electrically heated to 550 °C. Total pyrolysis time was 90 s although the pyrolysis reaction was completed in less than 50 s. The residence time of the pyrolysis vapors in the reactor pyrolysis zone has been estimated to be ~75 ms and is short enough that secondary cracking reactions in the quartz reactor are minimal. Mass spectral data from 15 to 450 Da were acquired by 22 eV electron impact ionization. With this system, both light gases and heavy tars are sampled simultaneously in real time. The mass spectrum of the pyrolysis vapor provided a rapid, semi-quantitative estimate of the molecular fragments. Results are presented as means across both biological and technical replicates per clone.

Measurement of chemical composition

The chemical composition of selected samples from individual lines of Clone OP-367 were analyzed for total lignin, structural sugars, extractives and ash based on protocols described by Browning (1967). Ethanol-soluble extractives were determined after soxhlet extraction with ethanol for 24 h. Total ash content was determined gravimetrically from samples that were ignited and then heated in an oven at 575 °C for 24 h. Acid hydrolysis lignin was determined gravimetrically on samples that were exhaustively ethanol extracted before the analysis. Each sample was subjected to 72% sulfuric acid treatment at 30 °C for 1 h before being autoclaved at 121 °C for an hour at an acid concentration of 4%. Acid-soluble lignin was determined spectrophotometrically at 205 nm. Sugar analysis was performed on the liquid fraction from the Klason lignin determination. The liquid fractions from the Klason determination were neutralized with sodium bicarbonate before analysis of sugars by high performance liquid chromatography (HPLC) with an Agilent 1100 liquid chromatography/mass spectrometer (LCMS) (Agilent Technologies, Palo Alto, CA). All analyses were carried out in duplicate and results are presented as means of the two experiments.

Gas chromatography–mass spectroscopy

Two leaves (LPI 10 ± 1) were sampled, quickly frozen in dry ice, shipped overnight to Oak Ridge National Laboratory and transferred to a −80 °C freezer until processed for analysis. Leaves were ground with a mortar and pestle in liquid nitrogen. About 300 mg frozen mass (FM) of tissue was placed in a tared vial to which sorbitol was added as an internal standard. Another 50 mg FM of sample was heated at 90 °C for 48 h to determine the FM/dry mass (DM) correction factor for each sample. The former samples were twice extracted with 5 ml of 80% aqueous ethanol at STP. The extracts were combined before analysis of metabolites related to lignin metabolism. After drying 2.5 ml of the 10-ml extract in a nitrogen stream, metabolites were analyzed as trimethylsilyl (TMS) derivatives by gas chromatography–mass spectrometry (GC–MS) using electron impact ionization (70 eV) (Gebre et al. 1998). Extracts were silylated with 0.5 ml *N*-methyl-*N*-trimethylsilyl-tri-fluoroacetamide (MSTFA) + 1% trimethyl-chlorosilane (TMCS) and 0.5 ml pyridine (Pierce Chemical, Rockford, IL),

heated for 60 min at 70 °C and 2 µl injected after two days into an HP 5890 Series II GC and 5972 MS (Hewlett-Packard, Avondale, PA), fitted with an HP-5MS (cross-linked 5% PH ME Siloxane) 30 m × 0.25 mm × 0.25 µm film thickness capillary column with operating conditions similar to Gebre et al. (1998). The injection port was configured in the splitless mode and operated at 250 °C and the ion source was operated at 300 °C. The MS was operated in the scan mode (50–550 Da) with 1.5 full scans s^{-1}. Peaks were quantified by area integration with the internal standard used to correct for sample loss and differences in derivatization efficiency. Metabolites associated with the lignin pathway, including precursors, pathway intermediates, branch point metabolites (e.g., *o*-coumaric acid) and glycosylated conjugates that accumulated in detectable quantities were analyzed. With the exception of phenylalanine and triandrin (*p*-coumaryl alcohol glucoside), which were integrated from the total ion chromatogram because of their sufficient abundance, all other metabolites were quantified by extracting a unique fragment that was characteristic of that metabolite to ensure the integrated peaks were free from co-eluting interfering metabolites. The metabolites quantified and the mass: charge ratio of fragments (m/z) extracted were: *trans*-cinnamic acid (205), *p*-coumaric acid (293), *o*-coumaric acid (293), caffeic acid (396), ferulic acid (338), sinapic acid glucoside (368), vimalin (methyl-triandrin) (236) and coniferin (324). All analyses were carried out in triplicate and results are presented as means of the three experiments.

Statistical analyses

The relationship between individual cell wall components among all transgenic lines and controls was examined by multivariate data analysis performed with Unscrambler version 7.8 (CAMO A/S, Trondheim, Norway). The mass spectra were normalized to the total ion current and 300 mass spectra variables (m/z values between 50 and 350) were used in the principal component analysis, which aided in the interpretation of the results through data reduction and visualization of the multivariate relationship among independent variables (Brereton 2003).

Based on pyMBMS multivariate results, chemical components of the cell walls for a selected number of transgenic and control lines from Clone OP-367 were analyzed for differences among lines within clone based on a simple one-way analysis of variance (ANOVA). When significant differences ($P \leq 0.05$) were detected among lines, single-degree-of-freedom orthogonal contrasts were used to test for differences between transgenic lines and non-transgenic controls. For the same plant materials, pairwise Student's *t* tests were used to compare GC–MS results between transgenic lines and their respective controls at $P \leq 0.05$.

Results and discussion

Apart from reduced herbivory, all transgenic lines across all clones were morphologically indistinguishable from their non-transgenic counterparts (Figure 2). Wood samples were collected and analyzed after Clones 50-197 and 311-93 had completed one full year of growth in the field and after Clone OP-367 had completed three full years of growth. A typical pyMBMS spectrum of the pyrolysis products from a poplar stem is shown in Figure 3. Major peaks in pyrolysis mass spectra, previously assigned (Evans and Milne 1987), are listed in Table 1. The complex nature of the pyrolysis mass spectrum makes a visual comparison of multiple mass spectra problematic. This difficulty arises not only because the mass spectra change individually, but because they change relative to differences in the amounts of cellulose, hemicellulose and lignin

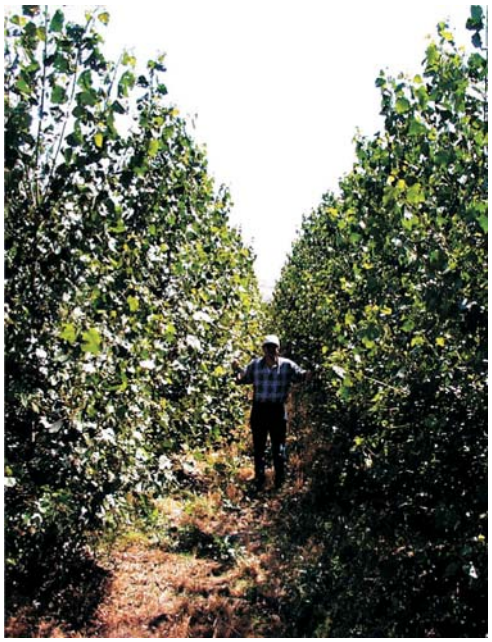

Figure 2. Transgenic plants were normal in appearance. The row at the left was planted exclusively with non-transgenic control Line N; the trees in the row at the right are transgenics. Note that because of increased herbivory, more light is passing through the canopy of the control trees.

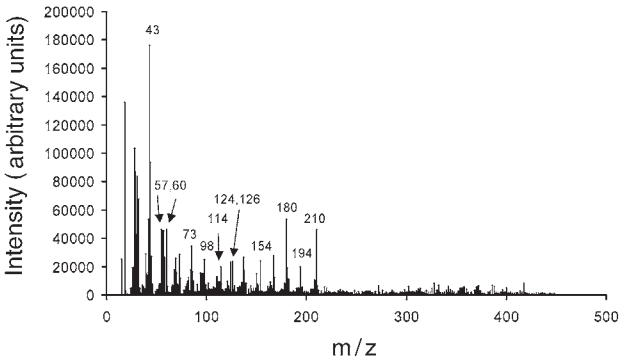

Figure 3. Typical mass spectrum of the pyrolysis products from a *Populus* stem sample. Major lignin peaks are shown and assignments are given in Table 1. Abbreviation: m/z = mass: charge ratio of fragments extracted.

Table 1. Mass spectrum peak assignments associated with pyrolysis molecular beam mass spectroscopy for hybrid poplar wood samples (based on Evans and Milne 1987). Abbreviation: m/z = mass:charge ratio of fragments extracted.

m/z	Assignment
57, 73, 85, 96,114	C5 Sugars
57, 60, 73, 98,126,144	C6 Sugars
94	Phenol
110	Catechol, resorcinol
120	Vinylphenol
122	Ethylphenol
124	Guaiacol
137 [1]	Ethylguaiacol, homovanillin, coniferyl alcohol
138	Methylguaiacol
150	Vinylguaiacol
154	Syringol
164	Allyl-+propenyl guaiacol
167 [1]	Ethylsyringol, syringylacetone, propiosyringone
168	4-Methyl-2, 6-dimethoxyphenol
178	Coniferyl aldehyde
180	Coniferyl alcohol, syringylethene
182	Syringaldehyde
194	4-Propenylsyringol
208	Sinapyl aldehyde
210	Sinapyl alcohol

[1] Fragment ion.

within individual cell walls. However, principal component analysis identified relationships among the peaks in the spectra.

pyMBMS Analysis of Clones 311-93 and 50-197

Principal component analysis was applied to the mass spectra obtained from each of 10 transgenic lines and three control lines for Clones 311-93 and 50-197. The scatter plots of the principal component 1 (PC1) scores versus the principal component 2 (PC2) scores of the pyMBMS spectra for each clone are shown in Figures 4a and 4b, respectively. The PC1 explained 39 and 42% of the total variance in the mass spectra, whereas PC2 explained 19 and 20% of the variance of Clones 311-93 and 50-197, respectively. Successive principal components (e.g., PC3, PC4) could not be assigned to known structural cell wall components (loadings appeared as noise) and so were not considered when interpreting differences in cell wall chemistry between the controls and transgenic lines. Individual trees could be identified in scatter plots for Family 50-197 but not for Clone 311-93, suggesting that the chemical composition varied more among ramets within Clone 50-197 than within Clone 311-93.

Analysis of the loadings associated with the PC1 and PC2 scores (data not shown) for Clone 311-93 revealed that most of the variation in the mass spectra was due to pyMBMS replicate-to-replicate variation, indicating the absence of chemical composition differences between samples. Analysis of the loadings associated with the PC1 and PC2 scores for Clone 50-197 (data not shown) indicated ramets within individual lines had minor differences in syringyl-to-guaiacyl (S/G) ratio (i.e., individual PC1 loadings displayed a slight shift to the right of the axis) or lignin content (i.e., individual PC2 loadings displayed a slight shift downward), or both. However, the pyMBMS analysis revealed no differences among the chemical constituents of the control trees and the transformed lines for either Clone 311-93 or Clone 50-197. That is, the individual multivariate data points for all controls resided within the 90% contour of the entire two-dimensional distribution.

pyMBMS Analysis of Clone OP-367

Principal component analysis was applied to the mass spectra obtained from Clone OP-367 control and transgenic lines. The scatter plot of the PC1 scores versus the PC2 scores is shown in Figure 5. Note that the *x*- and *y*-axes have expanded for OP-367 relative to 311-93 and 50-197, reflecting the wider range of cell wall chemistry values in the 3-year-old trees. The PC1 and PC2 explained 16 and 6% of the variance, respectively. In Figure 5, the symbols connected by lines forming a triangle are technical replicates from the same ramet and represent within-sample variation in the pyMBMS analysis. The small amount of variance explained by the first two PCs and lack of information in the subsequent PCs suggests that all of the OP-367 samples were very similar in chemical composition. However, although little variance was explained by the principal component analysis of the mass spectra, there were

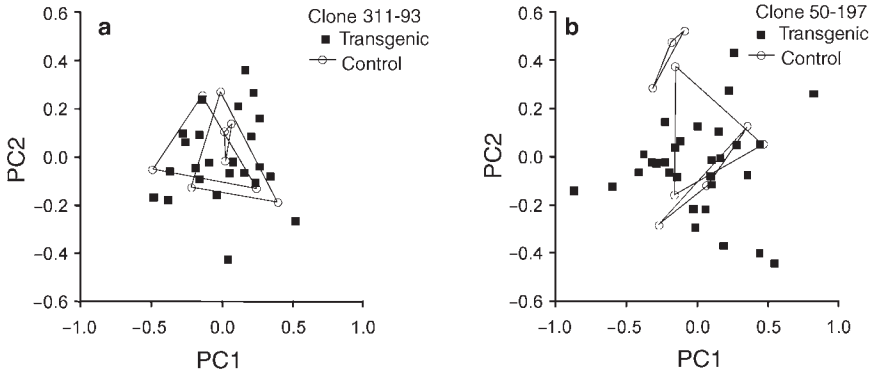

Figure 4. The principal component 1 (PC1) versus principal component 2 (PC2) plots of molecular beam mass spectrometry (MBMS) data for: (a) Clone 311-93 and (b) Clone 50-197. Values connected by lines forming triangles represent technical replicates of the same sample and indicate the typical error of the pyrolysis vapor MBMS analysis. For clarity, values of transgenic sample replicate runs are not connected by lines.

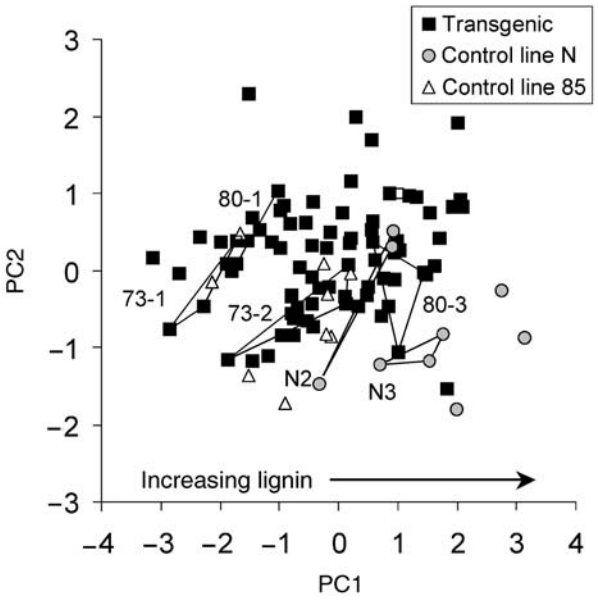

Figure 5. A principal component 1 (PC1) versus principal component 2 (PC2) plot of molecular beam mass spectrometry (MBMS) data for Family OP-367. Values connected by lines forming triangles indicate technical replicates of the same sample and indicate the typical error of the pyrolysis vapor MBMS analysis. For clarity, other values of replicate runs are not connected by lines. The labels near each triangle identify the samples selected for traditional chemical analyses.

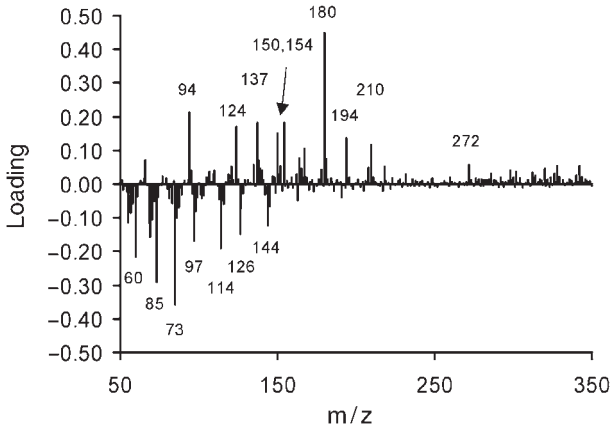

Figure 6. Loadings associated with principal component 1. Major lignin peaks are shown and assignments are given in Table 1. Abbreviation: m/z = mass:charge ratio of fragments.

slight differences in chemical composition detected among the different lines. For example, a clear separation along PC1 is observed between transgenic trees from Lines 73 and 80 and among the control trees Lines N-1, N-2 and N-3. There is no clear clustering of the transgenic trees with respect to the control trees from Line 85 indicating that cell wall chemistry of this non-transgenic control line is indistinguishable from that of the transgenic lines.

Analysis of the loading plot associated with PC1, shown in Figure 6, indicates that the greatest variance in the mass spectra is due to pyrolysis fragments that can be assigned to carbohydrates and lignin. The variables with higher positive loadings are associated with samples that have larger positive PC1 scores. The variables with positive loadings, m/z 94, 124, 137, 150, 154, 180, 194 and 210, are aromatic fragments that are released primarily during the pyrolysis of lignin (Table 1). The variables with larger negative loadings, m/z 60, 85, 73, 97, 114, 126 and 144, are fragments released during the pyrolysis of carbohydrate compounds and are associated with samples that have higher negative PC1 scores. Thus, the PC1 loadings indicate the samples having more positive PC1 scores have more lignin and samples having more negative PC1 scores have more structural carbohydrates. This was the basis for the subsequent wet chemistry and GC–MS analyses.

Traditional wet chemistry and GC–MS analyses

Cell wall components on a selected set of samples were analyzed by traditional methods of analytical chemistry (Browning 1967) to ascertain the magnitude of the changes in cell wall composition detected by the pyMBMS analysis. Two trees from each of two transgenic lines (73 and 80 as noted above) and from the control line (N), determined to have the greatest differences in chemical composition during the initial pyMBMS screen, were selected for traditional analysis. The results of the traditional chemical analysis for the selected samples are given in Table 2. There were significant minor differences in the contents of lignin, galactan and mannan between the control line and transgenic Line 80 (Table 3). Likewise, there were significant minor differences between transgenic Line 73 and the Line N control in lignin, arabinan and mannan contents. These differences in cell wall chemistry were always less than 1% and were always within the natural range of the tested cell wall components. These results agree with the overall pyMBMS multivariate analysis showing the control Line N has a slightly higher lignin content and slightly

Table 2. Mean chemical composition of selected transgenic and control lines from Clone OP-367. Percentages for structural components are given on an extractive-free basis. Total proportional dry mass per sample was not driven to closure (i.e., the total percentages do not sum to 100%).

Line	% Lignin	% Glucan	% Xylan	% Galactan	% Arabinan	% Mannan
N (control)	24.25	43.05	17.90	1.35	0.75	2.60
73	23.60	43.15	18.40	1.45	0.85	3.05
80	24.20	42.20	18.70	1.45	0.70	3.30

Table 3. Single-degree-of-freedom orthogonal contrasts from a one-way ANOVA for cell wall components based on two replicates per line. Statistical significance: ns = non-significant; * = $P \leq 0.05$; and ** = $P \leq 0.01$. Abbreviation: N = control line.

Line	% Lignin	% Glucan	% Xylan	% Galactan	% Arabinan	% Mannan
N versus 73	**	ns	ns	ns	*	**
N versus 80	**	ns	ns	**	ns	**

lower carbohydrate content than the transgenic lines. Similarly, examination of metabolites associated with lignin biosynthesis by GC–MS revealed a significant shift in a single lignin precursor in control Line N when compared with transgenic Lines 73 and 80 (Table 4). Coniferin was 4.9× higher in the transgenic replicates of Line 73 than in the Line N controls. Metabolite data are presented as the ratio of the means of the transgenic clones (73 or 80) over the controls (N) to provide a relative, differential response. In most cases there were no other differences among lignin precursor values for the control and either tested transgenic line.

In summary, among individual transgenic lines from three separate clones, there was generally no distinct clustering of transgenic poplars separate from the non-transgenic lines in any the PC1 versus PC2 score plots of the pyMBMS spectral data, indicating that the chemical composition of the structural cell wall components did not significantly vary between the control and transgenic poplars. There were slight differences detected after multivariate analysis of the pyMBMS spectra in some cell wall subcomponents in one non-transgenic control for Clone OP-367. The small differences in structural cell components detected by the pyMBMS experiment, confirmed by traditional analytical and GC–MS methods, was proportionately very small and was within the range of variants across biological samples and at the limits of variants detectable variation (i.e., < 1.0 relative percent or 24.25 versus 24.20 absolute percentage). It was not possible to specifically determine the cause of these subtle changes given the variability in propagation history among the tested lines, variation among biological replicates and variationg among technical replicates. Overall, our results indicate that the introduction of the Bt gene into the three different poplar clones leads to no significant unintended effects on cell wall chemistry.

Table 4. Comparative response of metabolites of selected Bt transgenic lines of Clone OP-367 relative to the control line N. Metabolites associated with the lignin pathway, including precursors, phenolic acids and glycosylated conjugates that accumulated to detectable levels were analyzed. Statistical significance: * = $P \leq 0.05$.

Metabolite	80 versus N	73 versus N
Phenylalanine	0.98	0.27
t-Cinnamic acid	1.46	1.03
p-Coumaric acid	0.81	1.12
o-Coumaric acid	1.04	1.06
Caffeic acid	0.75	1.48
Ferulic acid	0.98	1.18
Sinapic acid glucoside	0.81	1.37
p-Coumaryl alcohol glucoside	1.02	1.63
Vimalin	2.17	2.17
Coniferin	2.69	4.90 *

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