Molecular Level Lignin Patterns of Genetically Modified *Bt*-Maize MON88017 and Three Conventional Varieties Using Tetramethylammonium Hydroxide (TMAH)-Induced Thermochemolysis

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Bt-maize MON88017, its near-isogenic line DKC5143, and the two conventional varieties DK315 and Benicia were subjected to tetramethylammonium hydroxide (TMAH)-induced thermochemolysis to reveal molecular level lignin patterns. MON88017 is genetically modified to express the Cry3Bb1 protein aimed at the Western corn rootworm *Diabrotica virgifera virgifera*, a serious threat for European maize production. The results indicated that roots of the *Bt*-maize were characterized by a slightly enhanced total lignin content (by ~7%) compared to the near-isogenic line, whereas the molecular-based patterns, expressed by the relative fractions of *p*-hydroxyphenyl, guaiacyl, and syringyl breakdown products (P-, G-, and S-units, respectively) were virtually identical for both lines. No effects regarding either total lignin or molecular-based lignin patterns could be observed for leaves, indicating that biogenesis of lignin was not pleiotropically affected by the genetic modification. Significant differences for both total lignin and different lignin proxies existed between the conventional maize lines. Molecular level lignin analysis by means of TMAH-induced thermochemolysis is able to distinguish conventional maize varieties. Further work is necessary to evaluate lignin-related pleiotropic effects in genetically modified maize plants. The validation and application of a commonly accepted method for lignin analysis, capable of characterizing lignin at the molecular level, is a prerequisite.

KEYWORDS: Lignin; molecular characterization; thermochemolysis; genetically modified maize; MON88017

INTRODUCTION

The assessment of the nutritional value and safety of food and feed derived from genetically modified plants is an urgent challenge. As a consequence, a large body of literature is devoted to the nontarget effects and environmental fate of Cry proteins derived from *Bacillus thuringiensis* (*Bt*) and expressed by a variety of *Bt*-crop plants (*1*). In the European Union (EU), only *Bt*-maize varieties derived from the event

MON810 and expressing the Cry1Ab protein specific for *Lepidoptera* have been authorized for planting until now. Under the respective EU regulation, genetically modified plants have to be assessed on a case-by-case basis, meaning that every genetically modified plant has to be assessed individually. A novel variety with relevance for the European market is MON88017 with resistance against the Western corn rootworm (WCR) *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) through the expression of the Cry3Bb1 protein. The WCR is currently invading Europe (*2*) and not readily controlled with current plant protection practices. The Cry3Bb1 protein, derived from *B. thuringiensis* ssp. *kumamotoensis*, is highly specific for the WCR and expressed with around 24 and 12 μg g^{-1} Cry3Bb1 protein (referred to fresh weight) in leaves and stems, respectively (*3*). The MON88017 maize variety has been cultivated in the United States since 2003.

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Table 1. Total Lignin Content in Maize As Determined by Different Methods

compartment	method	total lignin[a] (%)	ref
whole plant	ADL[b]	2.8–5.3	Amon et al. (*10*)
whole plant (14 varieties)	Klason lignin	15 (averaged)	Jung and Sheaffer (*4*)
whole plant (14 varieties)	thioacidolysis	13 (averaged)	Jung and Sheaffer (*4*)
whole plant (14 varieties)	ADL	7 (averaged)	Jung and Sheaffer (*4*)
maize silages	ADL	8.2–10.4	Mechin et al. (*11*)
maize silages	ADL	4.4–7.5	Kruse (*12*)
maize silages	permanganate	3.4–4.5	Boever de et al. (*13*)
root	Klason lignin	8	Iritani and Arnold (*14*)
stover	permanganate	3.9	Wolf et al. (*9*)
whole plant (12 varieties)	ADL	10–16	Falkner et al. (*15*)
stem (hydroponic)	Klason lignin	12	our own unpublished results

[a] Referred to dry matter. [b] Acid detergent (cetyl trimethylammonium bromide) lignin (cellulose = ADF − ADL, ADF-acid detergent fiber (*12*).

The genetic modification of plants may result in pleiotropic effects, that is, alterations of one or many, micro- and macroscopic plant characteristics somehow brought about by the insertion of the transgenic materials coding for the novel protein and its regulatory sequences. Special emphasis has been put on the study of pleiotropic effects on lignin (*4, 5*). Lignin enhances the strength of fibrous tissues and improves water conduction through xylem tracheary elements. In addition, lignification reduces the degradability of cell walls by hydrolytic enzymes, providing resistance to pathogens and defense against ultraviolet radiation (see *6* and references cited therein). Lignin is produced by plants by linking together *p*-coumaric, coniferyl, and sinapyl alcohols to synthesize *p*-hydroxyphenyl (P), guaiacyl (G; 1-OH-2-methoxyphenyl), and syringyl (S; 1-OH-2,6-dimethoxyphenyl) lignin monomeric units, respectively. Thus, lignin comprises diverse subunits cross-linked by a multitude of C−C and C−O−C linkages.

Until now, there has been no single standard method to determine total lignin content. A multitude of methods are used instead, all of them having specific advantages and drawbacks (*7*). The chiefly applied, time-consuming "wet chemistry" methods to determine total lignin content include thioacidolysis, oxidation with permanganate, cupric oxide or (alkaline) nitrobenzene, and acetyl bromide assays, as well as the widely used Klason method (which yields a fraction insoluble in 72% sulfuric acid after pretreatment with ethanol/benzene) (*8*). Results obtained using different methods scatter widely for a given sample (*4*; see also Cornell Composting Homepage http://cwmi.css.cornell.edu/). A compilation of data from different sources on the total lignin content of maize as obtained by different methods is given in **Table 1**.

Molecular level characterization is more important than total lignin content in the investigation of the effects on herbivores, digestibility, and plant growth architecture, as well as soil organic matter stabilization by lignin transformation and/or decomposition processes after plant decay (*16*). The obviously contrasting decomposition rates of phenol-originating lignin moieties also determine the digestibility of maize substrates for biogas production (*10*). Consequently, there is a tremendous interest in developing and validating efficient methods for lignin analysis at the molecular level (*7*).

Tetramethylammonium hydroxide (TMAH)-induced thermochemolysis combined with GC-MS is a very efficient, highly sensitive method for revealing lignin breakdown units of the P-, G-, and S-type without time- and reagent-consuming sample preparation (*17* and references cited therein). Thermochemolysis occurs due to the basic character of the TMAH reagent, resulting

in the hydrolysis of ester and ether bonds, in particular, β-aryl ether linkages (*18*). TMAH-induced thermochemolysis is generally carried out at 500−600 °C for 3−5 s in the online mode, whereas the off-line approach—usually performed in sealed ampules—is characterized by lower temperatures (200−250 °C) and longer reaction times (90−120 min) (*17*). Thermally assisted chemolysis combines in situ hydrolysis of the biopolymer and methylation of acidic functional groups (carboxylic group, phenolic OH-groups) of the breakdown products, generating less polar analytes (methyl esters and methoxy compounds, respectively), which are easily accessible with GC. Thus far, TMAH-induced thermochemolysis has provided insights into the fate of lignin during fungal decay, mineralization, solubilization, and reactions with organic pollutants (*19* and references cited therein).

As described by Saxena and Stotzky (*5*), genetic modification of *Zea mays* L. with the *Cry1Ab* gene derived from *Bt* resulted in higher lignin contents for all three events Bt11, Bt176, and MON810 when compared to the corresponding near-isogenic lines, even in different hybrid backgrounds. These results were confirmed by Poerschmann et al. (*20*), albeit the enhancement of the total lignin content was shown to occur to a smaller extent, contrary to the findings of Jung and Sheaffer (*4*), where the presence of the inserted *Bt*-transgene was found not to alter lignin concentration in maize stover in commercial hybrids. The studies of Jung and Sheaffer (*4*) as well as Saxena and Stotzky (*5*) were based on common methods to determine total lignin, thus considering lignin as a "bulk". In contrast to the consideration of lignin as a total parameter, Poerschmann et al. (*20*) addressed the molecular dimension of lignin, which was revealed by applying offline thermochemolysis combined with GC-MS coupling. Continuing the work on lignin patterns of genetically modified Cry1Ab expressing maize, the *Bt*-maize MON88017 and the corresponding near-isogenic line DKC5134 were studied. Plant organs under investigation included roots and leaves of both the genetically modified and the near-isogenic lines. Beyond the comparison of these two lines, another objective of this study was to investigate the natural variation in lignin patterns, in particular, the diagnostic ratios of significant lignin breakdown products. For this reason, two additional varieties, DK315 and Benicia, were also studied.

MATERIALS AND METHODS

Chemicals and Samples. TMAH, as well as isotopically labeled [$^2H_2$]-phenanthrene and 2,2′-d_2-palmitic acid (both used as internal standards for quantitation purposes) were purchased from Supelco (Munich, Germany). The latter was used for the analysis and quantitation of fatty acids (unpublished procedures). Lignin (hydrolytic), considered as being of 100% purity for calibration purposes, was purchased from Aldrich (Munich, Germany). The MON88017 maize line and its near-isogenic counterpart DKC5143, as well as two conventional varieties DK315 and Benicia, were grown on a field-release site (see ref *3*). The soil, with an organic carbon content of 0.67−0.71% in the top layer (0−20 cm), can be classified as a pseudogleyic sandy loam. Maize was sown in early May 2005 in rows 0.75 m apart from each other, with a final plant density of 9−10 plants m^{-2}. Plants grew under identical conditions. Ten randomly selected plants of each variety were harvested at growth stage BBCH 75. Harvesting maize plants at similar growth stages is essential because lignin composition can change during plant development (*21*). Maize compartments studied included roots and two kinds of leaves: the first were leaves from the fourth elongated, above-ground internode (in what follows called "old" leaf), whereas the second were leaves from the fourth internode from the top of the stem (called "young" leaf). MON88017 was compared to its near-isogenic counterpart DKC5134 with regard to both total lignin content and molecular level lignin

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pattern. Likewise, the two conventional lines were compared to each other and also to the DKC5134 line (genetically modified and isogenic). The roots and leaves of the harvested maize plants were cut, homogenized, and freeze-dried. Roots were washed cautiously with distilled water to remove adhering soil matrix. After freeze-drying, samples from each plant of a given variety were combined and carefully homogenized. Eight equal subsamples (about 2 mg each) of each plant organ and each maize variety were used for thermochemolysis.

Thermochemolysis. Freeze-dried, homogenized maize matrix (about 2 mg) was digested with 200 μL of 25% (w/w) TMAH solution in methanol in flame-sealable glass ampules. The TMAH solution was prepared fresh daily to avoid reagent decomposition. Internal standard, 2,2′-d_2-palmitic acid, was added to the samples (1000 μg g^{-1} referred to dry biomass). Samples were placed under a stream of nitrogen, then set under vacuum, and flame sealed. Although the online method is less time-consuming, the offline method was used in this study because of its better precision and reproducibility (*20*). In addition, higher yields of lignin monomers can be obtained in the offline approach, rendering it more representative. Preliminary data obtained with the nondiscriminating pyrolysis approach (*22*) provide strong evidence that summing up the 15 most abundant TMAH breakdown products gives yields of ~13% referred to dried lignin matter (results not detailed here). The most important cause of the poor yield proved to be the release of small fragments (CO_2, CO, H_2O) along with monocarboxylic acids (acetic, propionic), all of which have no diagnostic value. In this study, thermochemolysis was conducted by rapid heating of the sealed ampules to 220 °C and maintaining this temperature for 120 min. The TMAH-reacted samples were cooled to room temperature and extracted with benzene (containing [^{2}H$_{10}$]-phenanthrene internal standard at a concentration of 1000 μg g^{-1} referred to the freeze-dried biomass), followed by an aliquot of 1 μL being injected into the GC using pulsed splitless injection mode. TMAH-induced breakdown products (see **Table 2**) were detected in their methylated form.

GC-MS. GC-MS analyses were performed using an Agilent HP5973A/HP 6890 system (Waldbronn, Germany), fitted with a split/splitless injector and a 30 m × 0.25 mm i.d. × 1.2 μm film thickness HP-5 ms capillary column. A conventional split/splitless liner, deactivated by silanization prior to use, was applied. A retention gap (2 m × 0.32 mm i.d. deactivated fused silica) was connected to the head of the column to protect the stationary phase. The retention gap was changed after eight runs, typically each working day. The same replacement interval was maintained for the deactivated liner in the GC injector. The oven temperature was typically programmed from 40 °C (held for 2 min) to 290 °C at a rate of 12 °C min^{-1}, which was then held for 5 min. The following MS operating conditions were used: 70 eV ionization potential of the electron impact source, 230 °C ion source temperature, and data acquisition in full scan mode covering a range from 33 to 420 amu. Lignin breakdown products were identified by their relative retention data and by comparison with mass spectra from the NBS library as described in Filley et al. (*18*), Vane (*23*), and Frazier et al. (*24*). Quantitation was performed using the internal standard method and diagnostic ions listed in **Table 2**. Quantitation of individual breakdown products was based on the ratios of the peak areas of the selected ions (underlined in **Table 2**) to that of the diagnostic ion $m/z = 188$ amu of the internal standard. The total lignin content of the maize compartments under study (referred to biomass) was calculated as the ratio of the sum of the diagnostic ion abundances of all lignin breakdown products to the internal standard diagnostic ion. Calibration was performed using the lignin standard purchased from Aldrich (assuming 100% lignin purity of the latter).

Statistics. The significance of the differences between the mean results obtained for transgenic, isogenic, and commercial maize varieties along with their ratios was evaluated by a one-way analysis of variance (ANOVA), followed by Fisher's least significant difference test (LSD, $p = 0.01$; SigmaStat 2.0, SPSS Inc., Chicago, IL). Beyond one-way analysis of variance, the results were treated by the multivariate statistic of principal components analysis (PCA). The data set was condensed into a small collection of linear relationships of two principal components (PC), which described most of the variation in the data. On this basis, the PCs can be used to identify which combinations of variables are associated with each other and to calculate the degree to

Table 2. Major Thermochemolysis Products from Maize Stems and Leaves with Their Diagnostic Fragments/Molecular Ions

identification	ID code[a]	fragment ion(s)/molecular ion[b]
methoxystyrene	P3	119/134
1,2-dimethoxybenzene	G1	123/138
3,4-dimethoxytoluene	G2	137/152
4-methoxybenzaldehyde	P4	92/135/136
1,2,3-trimethoxybenzene	S1	153/168
4-methoxyacetophenone	P5	135/150
4-methoxybenzoic acid methyl ester	P6	135/166
3,4-dimethoxystyrene	G3	91/149/164
3,4-dimethoxybenzenemethanol methyl ester	G20	167/182
1-(4-methoxyphenyl)-2-propanone	P22	149/164
3,4,5-trimethoxytoluene	S2	139/167/182
3,4-dimethoxybenzaldehyde	G4	151/165/166
1-(3,4-dimethoxyphenyl)-1-propene	G21	163/178
4-methoxybenzenepropanoic acid methyl ester	P12	121/194
3,4-dimethoxyacetophenone	G5	137/165/180
1-(3,4-dimethoxyphenyl)-2-propanone	G22	151/194
3,4-dimethoxybenzoic acid methyl ester	G6	165/181/196
cis-3-(4-methoxyphenyl)-3-propenoic acid methyl ester	P17	133/161/192
3,4,5-trimethoxybenzaldehyde	S4	125/181/196
cis-1-(3,4-dimethoxyphenyl)-2-methoxyethylene	G7	151/179/194
trans-1- (3,4-dimethoxyphenyl)-2-methoxyethylene	G8	151/179/194
cis-3-methoxy-1-(3,4-dimethoxyphenyl)-1-propene	G9	193/208
cis-1-(3,4-dimethoxyphenyl)-1-methoxy-1-propene	G10	165/193/208
trans-1-(3,4-dimethoxyphenyl)-1-methoxy-1-propene	G11	165/193/208
trans-3-(4-methoxyphenyl)-3-propenoic acid methyl ester	P18	133/161/192
3,4,5-trimethoxyacetophenone	S5	139/195/210
3,4,5-trimethoxybenzoic acid methyl ester	S6	195/211/226
3-(3,4-dimethoxyphenyl)propanoic acid methyl ester	G12	151/224
trans-1-(3,4-dimethoxyphenyl)-3-methoxyprop-1-ene	G13	91/177/208
cis-1-(3,4,5-trimethoxyphenyl)-2-methoxyethylene	S7	181/209/224
cis-3-(3,4-dimethoxyphenyl)-3-propenoic acid methyl ester	G17	191/207/222
trans-1-(3,4,5-trimethoxyphenyl)-2-methoxyethylene	S8	181/209/224
threo-1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane	G14	166/181/270
erythro-1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane	G15	166/181/270
cis-1-(3,4,5-trimethoxyphenyl)-methoxyprop-1-ene	S10	195/223/238
trans-3-(3,4-dimethoxyphenyl)-3-propenoic acid methyl ester	G18	191/207/222
trans-1-(3,4,5-trimethoxyphenyl)-3-methoxyprop-1-ene	S13	223/238
cis-1-(3,4-dimethoxyphenyl)-1,3-dimethoxyprop-1-ene	G16	176/207/238
threo-1-(3,4,5-trimethoxyphenyl)-1,2,3-trimethoxypropane	S14	211/300
erythro-1-(3,4,5-trimethoxyphenyl)-1,2,3-trimethoxypropane	S15	211/300

[a] Analytes listed according to elution sequence on nonpolar DB-5 ms stationary phases. [b] Quantitation ions underlined.
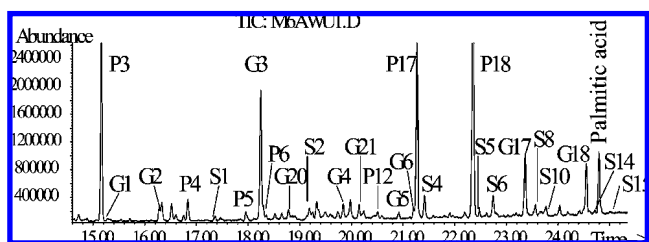

Figure 1. Total ion current (TIC) chromatogram of thermochemolysis breakdown products of the root of the genetically modified maize line MON88017. Peaks: see **Table 2**.

which they impact the system under study (factor loadings > |0.9|). One objective was to differentiate and plot clusters of closely related genetically modified, near-isogenic, and conventional maize varieties, as well as diagnostic ratios. The second objective was to recognize the most sensitive plant organs (roots, old leaf, and young leaf) along with their proxies for the separation of the four maize varieties under study. SPSS statistical software version 10.0 (SPSS Inc.) was used for these purposes.

RESULTS

Identification and Quantitation of Lignin Breakdown Products. Thermochemolysis breakdown products of lignin are depicted in **Figure 1** using the root of the genetically modified maize line MON88017 as an example. Peak labels are explained in **Table 2**, whereas detailed structural information is given in
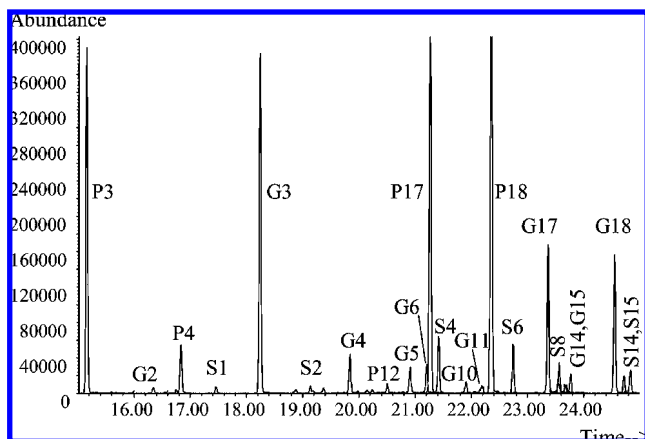
Figure 2. Sum of selected ions of thermochemolysis breakdown products from the root of the genetically modified maize line MON88017. Peaks: see **Table 2**.

Poerschmann et al. (*20*). Monomers of the P-type, along with the guaiacyl breakdown product G3, accounted for the most abundant peaks. The P17 and P18 units were esterified at the γ-position of the phenylpropane side chain in the lignin molecule, with the *p*-coumaric acid assumed to be preferentially esterified by syringyl units (*16*). Cinnamyl alcohols, including *p*-coumaryl, coniferyl, and sinapyl alcohol (see P9, P13, G9, G13, S9, and S13; **Table 2**), could not be detected in the root samples. As expected, the "grassy" maize lignin (angiosperm) was composed of P-, G-, and S-monomers, whereas woody angiosperms consist entirely of G- and S-units. Syringyl constituents found in maize lignin are known to facilitate overall lignin degradation (*25*).

Unambiguous peak assignments to the individual breakdown products along with their quantitation are error-prone and cumbersome if total ion chromatograms (TIC) are used for these purposes (see **Figure 1**). This is due to the large number and variety of products generated by thermochemolysis, including breakdown products originating from carbohydrates and lipids, which leads to coelution of peaks and hinders detection of minor components. To circumvent this obstacle, selected diagnostic ions were used to "extract" the lignin-derived target analytes out of the TIC, allowing more reliable quantitation. This methodology, as applied in this study, is exemplified in **Figure 2**, which depicts the sum of selected diagnostic ions for the labeled lignin breakdown products. For a better comparison, **Figures 1** and **2** are based on the same data file. Unambiguous structural assignment of the breakdown products was made possible by tracing the diagnostic ions in the corresponding retention time intervals. Beyond that, relationships between chemical structure and gas chromatographic retention behavior were used (see listed retention indices given in http://webbook.nist.gov/chemistry/form-ser.html) to confirm structural assignments brought about by mass spectroscopic identification. Attention should be paid to the structural assignment of the isomers, as wrong peak assignment obviously results in flawed proxies. As an example, the lignin-derived breakdown product G1 (1,2-dimethoxybenzene) should not be confused with 1,3-dimethoxybenzene originating from carbohydrates. Likewise, S1 (1,2,3-trimethoxybenzene) should be distinguished from the generally very abundant thermochemolysis product 1,2,4-trimethoxybenzene, which might originate from both carbohydrates and tannins.

Lignin Alteration Proxies. To characterize lignin sources and lignin decay in soils and sediments, the yields of lignin monomers along with the ratios of diagnostic thermochemolysis breakdown products have proven to be essential. In this study, both type-specific proxies (S/G, S/P, G/P) and proxies focused on individual breakdown products [G6/G4, S6/S4, G6/(G14+G15), S6/(S14+S15)] were examined. Diagnostic proxies were originally developed for studying the lignin fraction in stabilized humic organic matter (*27* and references cited therein). As an example, large abundances of *p*-coumaric acid (P-type) and ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, G-type) in soil allow distinguishing between lignin from nonwoody and woody sources. In this study, advantage was taken of the following proxies to characterize lignin in the different compartments of the four maize varieties under study:

1. The ratio of 3,4-dimethoxybenzoic acid methyl ester (ME) to 3,4-dimethoxybenzaldehyde (G6/G4; see **Table 2**) and the ratio of 3,4,5-dimethoxybenzoic acid ME to 3,4,5-dimethoxybenzaldehyde (S6/S4) refer to the relative decomposition state of guaiacyl and syringyl lignin monomers (*28*). Generally, the higher this ratio, the higher the susceptibility toward oxidation (*23*).

2. The G6/(G14+G15) and S6/(S14+S15) ratios are determined by the lignin side-chain oxidation. The G(S)14 and G(S)15 units point to moieties with a complete alkyl chain (see structures given in ref *20*). However, these ratios might be biased because the TMAH-induced release of G(S)14 and G(S)15 is dependent on chemical factors (*18*). Likewise, G(S)4 is assumed to be derived from the decomposition of G(S)14 and G(S)15. Consequently, they may share the same source. Breakdown products with trimethoxylated 3-carbon side chains attached, including G(S)14 and G(S)15, indicate that TMAH-induced thermochemolysis is a relatively mild treatment.

3. The P/G ratio points to "grassy" characteristics of lignin (*26*).

4. The S/G ratio refers to "angiosperm" characteristics, as well as to the selective degradation of syringyl-based lignin units. Less condensed syringyl moieties are characterized by higher oxidative reactivity relative to guaiacyl units (*29*).

Type-Specific Lignin Proxies of the Genetically Modified and the Corresponding Near-Isogenic and Conventional Lines. Table 3 summarizes the total lignin content along with type-specific proxies for all varieties under investigation. The results obtained for roots revealed that the total lignin content of the *Bt*-variety MON88017 was higher by ∼7% compared to its near-isogenic counterpart DKC5143, with the difference being statistically significant. On the other hand, the other three proxies (S/G, S/P, and G/P) revealed no significant differences. The molecularly determined lignin pattern of the *Bt*-variety was characterized by lower fractions of syringyl-type breakdown products compared to the P-type surrogates (by ∼5%). The former are generally regarded to be more susceptible to oxidation due to the presence of the OH-group in position 5 of the aromatic nucleus. Significantly enhanced G-type fraction, as observed for stems of the genetically modified line MON810 expressing the Cry1Ab protein (*20*), was not observed in this study. Total lignin and two of the three proxies studied were also significantly different between the two conventional maize varieties DK315 and Benicia, with almost 20% less total lignin in the former as compared to the latter.

The total lignin content and the molecular distribution of lignin breakdown products of maize roots might be affected by soil attached to the roots. However, the impact of soil-originating lignin toward the molecular pattern could be neglected in this case due to the low organic carbon content of the soil (∼0.7%).

The data for leaves of the genetically modified maize line and its near-isogenic counterpart indicated that the lignin patterns

Table 3. Proxies for Lignin Patterns of the Genetically Modified, Near-Isogenic, and Two Conventional Maize Lines for Root and "Old" Leaf and "Young" Leaf Compartments (Total Lignin in Percent Referred to Dry Biomass, w/w)

compartment	proxy	MON88017 modified	DKC5143 near-isogenic	modified/ isogenic	DK315 conventional	Benicia conventional
root	total lignin (%)	9.13 d[a]	8.53 c	1.07 (%/%) a	8.29 b	9.97 e
	S/G	0.342 a	0.349 a	0.98 a	0.323 a	0.348 a
	S/P	0.157 a	0.165 a	0.95 c	0.196 b	0.158 a
	G/P	0.459 ab	0.473 b	0.97 d	0.609 c	0.454 a
old leaf	total lignin (%)	4.86 d	4.70 c	1.03 (%/%) a	4.48 b	5.04 e
	S/G	0.495 cd	0.478 bc	1.03 e	0.460 b	0.388 a
	S/P	0.611 ab	0.618 ab	0.99 d	0.637 bc	0.592 a
	G/P	1.235 b	1.293 c	0.96 a	1.387 d	1.526 e
young leaf	total lignin (%)	4.52 c	4.55 cd	0.99 (%/%) a	4.31 b	4.76 e
	S/G	0.464 a	0.476 a	0.97 c	0.493 ab	0.471 a
	S/P	0.696 a	0.692 a	1.00 c	0.786 b	0.703 a
	G/P	1.502 a	1.453 a	1.03 c	1.598 b	1.495 a

[a] Means within rows for each proxy of lignin patterns followed by the same letter were not significantly different as determined by one-way ANOVA followed by the least significant difference (LSD, $p = 0.01$).
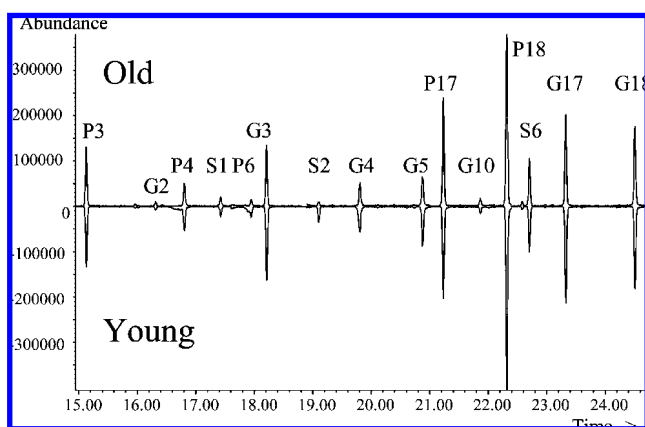

Figure 3. Thermochemolysis breakdown products of leaves from the genetically modified maize line MON88017: (top) old leaf; (bottom) young leaf. Data are presented as the sum of diagnostic ions (see **Table 2**) in defined retention time intervals (mirror presentation). Peaks are normalized to identical abundances of the internal standard.

of the old and young leaves for a given line were similar. This finding is exemplified in **Figure 3**, showing the two patterns in a mirror presentation for the *Bt*-maize line. A comparison of the root lignin pattern with those obtained for the leaves (**Figures 2** and **3**; genetically modified line in both cases) revealed that the root pattern was more complex, with significantly higher abundances of P-type breakdown products. Generally, the S/G, S/P, and G/P diagnostic ratios, as well as the patterns of the individual breakdown products (see **Figure 3**), were almost identical between the genetically modified line and its near-isogenic counterpart.

For the old leaf compartment, the lignin fraction in MON88017 was somewhat higher again (by ~3%). The G/P ratio was significantly different from that of the near-isogenic counterpart (**Table 3**). The majority of the corresponding diagnostic ratios (S/G, S/P, G/P), as well as the total lignin content, was significantly different for the two conventional varieties DK315 and Benicia. The molecularly based lignin pattern of the *Bt*-maize was characterized by lower G/P ratios (by ~4%). Hence, the minor enhancement of the total lignin content was due to slightly enhanced S- and P-type contributions.

The results obtained for the young leaf compartment were generally very similar to those obtained for the old leaf for both the *Bt*-maize and the near-isogenic line. The total lignin content and the type-specific proxies (S/G, S/P, G/P) were almost

identical for both lines. No statistically significant differences were found between the four proxies (lignin, S/G, S/P, and G/P) for the young leaf compartment (**Table 3**). Data accumulated for the three compartments under study provided strong evidence that the differences in the total lignin content and in the molecular composition were minor (root) or negligible (both kinds of leaves) when plant organs of the genetically modified and the near-isogenic lines were compared.

Determination of the type-specific lignin patterns of the conventional lines DK315 and Benicia aimed at establishing whether the total lignin content and/or the molecular lignin patterns could be used to distinguish between different maize varieties. This possibility is very interesting, especially considering that the conventional line DK315 produced significantly different proxies in comparison to the other varieties (**Table 3**). The data listed indicate that total lignin content might be a useful criterion in this context. For example, the Benicia variety was characterized by the highest lignin content among all varieties studied, whereas the DK315 variety was characterized by the lowest values. This statement is valid for all three plant organs studied. On the other hand, the type-specific ratios listed in **Table 3** were not specific enough to distinguish between the maize varieties. The Benicia variety tended to have lower S-fractions for the old leaf compartment, but the difference was significant only for the S/G ratio.

Due to the low applicability of the type-specific molecular proxies including S/G, S/P, and G/P ratios, the molecular proxies based on individual monomeric breakdown products, including G(S)6/G(S)4 and G(S)6/(G(S)14+G(S)15) ratios, were considered to reveal differences between the varieties under study.

Proxies Based on Individual Breakdown Products To Distinguish between Maize Varieties. Table 4 lists the G(S)6/G(S)4 proxies, which describe the oxidative susceptibility (acid/aldehyde ratio) for all varieties under study. Comparisons of the results obtained for MON88017 and DKC5143 provided strong indication that the oxidative stability of the maize did not change due to the insertion of the *Bt*-gene. Statistical analysis of the data revealed significant differences between the genetically modified and the near-isogenic lines with respect to the G6/G4 ratio in roots and the S6/S4 ratio in old and young leaves (**Table 4**). The S6/S4 ratios for both leaf compartments were significantly lower for the two conventional varieties. In general, the G(S)6/G(S)4 proxies were significantly different for the conventional varieties in nearly all plant organs.

Lignin Patterns of Genetically Modified *Bt*-Maize MON88017

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Table 4. G(S)6/G(S)4 Proxies for All Maize Varieties under Study

compartment	proxy	MON88017 modified	DKC5143 near-isogenic	DK315 conventional	Benicia conventional
root	G6/G4	1.05 a[a]	1.10 b	1.11 bc	1.19 d
old leaf		1.16 d	1.05 ad	1.11 bc	1.03 a
young leaf		1.02 a	1.01 a	1.04 a	0.93 a
root	S6/S4	1.24 b	1.22 b	1.25 b	1.18 a
old leaf		7.3 d	6.5 c	4.1 b	4.0 a
young leaf		9.2 c	10.6 d	4.2 b	3.6 a

[a] Means within rows for each proxy followed by the same letter were not significantly different, as determined by one-way ANOVA followed by the least significant difference test (LSD, $p = 0.01$).
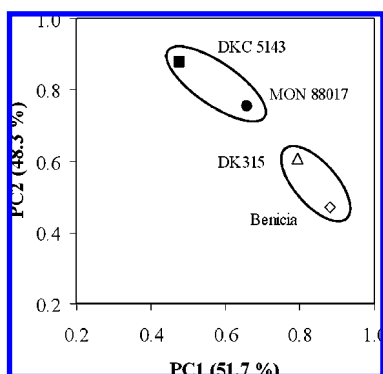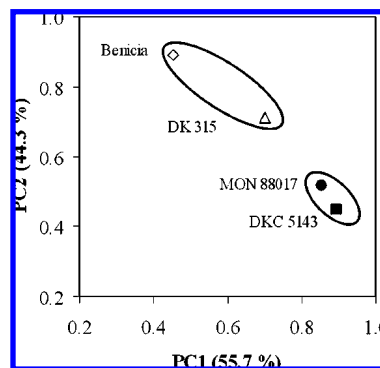

Figure 4. Ordination plot of the PCA separation of genetically modified (MON88017), near-isogenic (DKC5143), and conventional (DK315, Benicia) maize varieties by the S6/S4 proxy for roots, old leaf, and young leaf.

Table 5. G(S)6/(G(S)14+G(S)15) Proxies for All Maize Varieties under Study

compartment	proxy	MON88017 modified	DKC5143 near-isogenic	DK315 conventional	Benicia conventional
root	G6/(G14+G15)	1.4 b[a]	1.2 a	5.5 d	3.5 c
old leaf		0.27 bc	0.33 d	0.25 b	0.21 a
young leaf		0.32 bc	0.29 b	0.36 d	0.19 a
root	S6/(S14+S15)	0.68 a	0.65 a	3.1 ab	3.9 c
old leaf		3.3 b	2.9 a	100 d	20 c
young leaf		4.2 b	4.0 a	100 d	15 c

[a] Means within rows for each proxy followed by the same letter were not significantly different, as determined by one-way ANOVA followed by the least significant difference test (LSD, $p = 0.01$).

PCA for the S6/S4 proxy (**Figure 4**) revealed a clear separation of MON88017 and DKC5143 on the one hand and DK315 and Benicia on the other hand. The distinctive clusters depicted in **Figure 4** indicated that the S6/S4 proxy might be used in differentiating between maize varieties. This classification was further elucidated by another PCA to get detailed information on the plant organs responsible for this differentiation. Extremely high factor loadings of PC 1 (0.965 for old leaf and 0.975 for young leaf) explained 63.6% of the variance, and PC 2 with a factor loading of 0.965 for roots explained a further 34.3%. Thus, the two components accounted for 97.7% of the total variance, which indicated that all plant organs had nearly the same contribution to distinguishing maize varieties under study from each other.

Table 5 lists the G6/(G14+15) and S6/(S14+15) ratios, both related to side-chain oxidation of the lignin breakdown product. In the case of the G6/(G14+15) ratio, significant differences between the genetically modified and the corresponding near-isogenic line were observed for the root and old leaf compartments. For the S6/(S14+15) proxy, such differences were observed for the old leaf and young leaf compartments.

Figure 5. Ordination plot of the PCA separation of genetically modified (MON88017), near-isogenic (DKC5143), and conventional (DK315, Benicia) maize varieties by the S6/(S14+S15) proxy for roots, old leaf, and young leaf.

Roots of both conventional varieties were characterized by significantly higher oxidative susceptibility of the C$_3$-side chains, as indicated by the G6/(G14+15) ratio. Likewise, both leaf compartments were significantly different due to high oxidative susceptibility, as evidenced by the large S6/(S14+S15) ratios.

The findings obtained by one-way ANOVA were confirmed by PCA of the S6/(S14+15) proxy. **Figure 5** provides strong indication that the conventional lines Benicia and DK315 could be clearly distinguished from MON88017 along with its near-isogenic line DKC5143. Thus, the S6/(S14+15) proxy might be another potential candidate to differentiate between maize varieties. The separation of maize varieties was influenced by factor loadings of 0.925 for old leaf and 0.969 for young leaf (PC 1, 64.0%), and a factor loading of 0.961 for roots (PC 2, 34.3%). The two components accounted for 98.3% of the observed variance, proving once again that all plant organs influenced the differentiation of the maize varieties under study.

DISCUSSION

The data on total lignin content and different lignin proxies show no clear significant difference between the genetically modified maize line MON88017 and its near-isogenic line DKC5143. On the contrary, this study provides strong evidence for the absence of a pleiotropic effect with respect to lignin due to the insertion of the transgenic cassette encoding for the Cry3Bb1 protein to combat *Diabrotica virgifera virgifera* and the CP4EPSPS protein and the corresponding regulatory sequences present in MON88017. However, roots of the *Bt*-maize were characterized by slightly enhanced total lignin content compared to the corresponding near-isogenic line. This might be related to the fact that the toxin is chiefly expressed in the root, but might also be based on a hybrid × environment interaction that has already been observed in maize (*4*).

These findings are in some contrast to the pleiotropic effects (higher total lignin content for the *Bt*-varieties) observed for the different Cry1Ab expressing lines Bt176, Bt11, and MON810 (*5, 20*). At present, a comparison of the findings presented in this paper with corresponding data obtained in other laboratories with the genetically modified maize MON88107 expressing the Cry3Bb1 protein (with respect to both total lignin content as well as molecular lignin patterns in the transgenic and isogenic lines) is not possible due to lack of published data. The only compositional study on MON88017 is that of McCann et al. (*30*), who compared the grain and forage composition of this *Bt*-maize to a range of conventional varieties, using different chemical parameters, among them fiber, total FA content, and

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amino acids. There were only single cases of statistically significant differences between MON88017 and the control, and all of these fell within the normal range of the conventional hybrids. Taken together, this indicates a substantial equivalence of this *Bt*-maize line to commercially available conventional maize varieties. This has also been reported by Jung and Sheaffer (*4*) for other *Bt*-maize lines.

The two conventional maize lines under investigation, DK315 and Benicia, on the other hand, exhibited statistically significant differences for both total lignin and different lignin proxies. This underscores the necessity of taking into account the normal variation between different maize lines in the investigation of potential differences between genetically modified varieties and their near-isogenic lines. Slight and also statistically significant differences might not be of biological relevance, as long as these fall within the normal range of the assessed parameters (*30*).

This work was centered around TMAH-induced thermochemolysis, which has been largely neglected in lignin analysis of plants (in contrast to studies of humic organic matter). In contrast to organic geochemistry, where thermochemolysis has developed into a very useful tool to study the stabilization of soil/sediment organic carbon, transformation of plant residues into stabilized organic matter, and related objectives, the characterization of plant materials has largely been performed by traditional bulk techniques lacking molecular information. We hope that the promising results obtained in this study will foster this research field. The obvious gaps in knowledge could be overcome by a round-robin test using genetically modified and corresponding near-isogenic lines. A prerequisite would be to utilize methods that allow the determination of both the total lignin content and the molecular patterns of the samples under study. TMAH-induced thermochemolysis seems to be the best suited for this purpose, as the alternative method, CuO oxidation, suffers from serious drawbacks and limitations (see Introduction).

In summary, TMAH-induced thermochemolysis was shown to be a very useful tool to address lignin composition on the molecular basis. Deeper insight into other topics (e.g., lignin synthesis pathways, the fate of lignin in soil after plant decay, or the natural variability in a large range of different maize lines) might be gained by using ^{13}C-labeled TMAH, as proposed by Filley et al. (*18*). Isotopically labeled TMAH allows distinction between aromatic methoxy groups originating from methoxyl functionalities and methoxy groups originally present as hydroxyl functionalities (e.g., 5-OH guaiacyl nuclei in maize as revealed by thioacidolysis and syringyl units) and might prove to be an interesting tool in further research.

ACKNOWLEDGMENT

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