Metabolite Profiling of Maize Kernels—Genetic Modification versus Environmental Influence

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Ⓢ Supporting Information

ABSTRACT: A metabolite profiling approach based on gas chromatography−mass spectrometry (GC-MS) was applied to investigate the metabolite profiles of genetically modified (GM) Bt-maize (DKC78-15B, TXP 138F) and Roundup Ready-maize (DKC78-35R). For the comparative investigation of the impact of genetic modification versus environmental influence on the metabolite profiles, GM maize was grown together with the non-GM near-isogenic comparators under different environmental conditions, including several growing locations and seasons in Germany and South Africa. Analyses of variance (ANOVA) revealed significant differences between GM and non-GM maize grown in Germany and South Africa. For the factor genotype, 4 and 3%, respectively, of the total number of peaks detected by GC-MS showed statistically significant differences ($p < 0.01$) in peak heights as compared to the respective isogenic lines. However, ANOVA for the factor environment (growing location, season) revealed higher numbers of significant differences ($p < 0.01$) between the GM and the non-GM maize grown in Germany (42%) and South Africa (10%), respectively. This indicates that the majority of differences observed are related to natural variability rather than to the genetic modifications. In addition, multivariate data assessment by means of principal component analysis revealed that environmental factors, that is, growing locations and seasons, were dominant parameters driving the variability of the maize metabolite profiles.

KEYWORDS: metabolite profiling, gas chromatography, Roundup Ready maize, Bt-maize, safety assessment, natural variability

■ INTRODUCTION

To date, genetically modified (GM) maize accounts for nearly 30% of the worldwide planted maize area.[1,2] Genetic engineering is being employed to improve the agronomic properties as well as the nutritional value of maize. However, prior to the placing of GM crops on the market, comprehensive safety assessments are required. A key element in the safety assessment of GM crops is the concept of "substantial equivalence" originally introduced in 1993 by the OECD.[3] This approach, which is based on a comparative determination of similarities and differences between the GM crop and an appropriate conventional counterpart, has been taken up in the joint FAO/WHO report on safety aspects of GM foods.[4] In a recent guidance document, the European Food Safety Authority (EFSA) suggested the use of non-GM isogenic varieties for vegetatively propagated crops and the use of genotypes with a genetic background as close as possible to the GM crop for sexually propagated crops.[5]

Regarding the required chemical characterization, safety assessment procedures currently applied to GM crops are primarily based on targeted compositional analyses of common crop-specific compounds; however, there are also suggestions to limit the investigations to potentially deleterious constituents.[6] Several studies have been conducted demonstrating compositional equivalence between GM maize and the conventional comparators.[7] Such targeted approaches may have limitations in detecting unintended effects in GM organisms due to their biased character.[8] Therefore, nontargeted metabolite

profiling techniques are being discussed as additional tools for the safety assessment of GM crops as they may increase the chance to detect metabolic changes not intended by the genetic modification.[8−14] In recent years, several profiling-based studies have been carried out on comparative analyses of GM crops including GM maize and the respective non-GM counterparts.[15] These studies revealed some statistically significant metabolic differences between GM and non-GM maize.[16−19] However, the maize materials investigated were not assessed under different environmental conditions; that is, potential effects of different growing locations and/or seasons on the maize metabolites were not considered. In its guidance documents on the risk assessment/safety evaluation of GM organisms, the European Food Safety Authority (EFSA) pointed out that comparative analyses should not be restricted to the GM crop and the respective conventional comparator, but metabolites should also be assessed in the light of natural variability that is inherent in conventionally bred crops.[4,20,21]

The crop metabolite phenotype is mainly defined by the genetic background (e.g., different cultivars), the breeding strategy (e.g., conventional crossing, genetic engineering, mutation breeding), the environmental conditions (e.g., growing location, season), the crop management systems used (irrigation, low- or

high-input systems), and the maturity status of the crop at harvest. The pronounced impact of these factors on the natural variability of crop metabolites has been demonstrated both by targeted analytical approaches[22,23] and by nontargeted metabolite profiling procedures.[24−27]

The aim of the present study was the application of a capillary gas chromatography−mass spectrometry (GC-MS) metabolite profiling to two sets of transgenic maize: (a) insect-resistant *Bacillus thuringiensis* (Bt) maize and (b) Roundup Ready maize tolerant to the herbicide glyphosate, and their respective conventional counterparts grown in South Africa and Germany. For the comparative investigation of the impact of genetic modifications versus environmental influences on the maize metabolite profiles, GM and non-GM maize samples were grown together under different environmental conditions, including different growing locations and seasons. Multivariate and univariate data analyses were conducted to assess the metabolic differences due to the genetic modification in light of the inherent natural metabolic variability.

■ MATERIALS AND METHODS

Sample Material. *South African Field Trials.* The transgenic Bt hybrid variety DKC78-15B (hybrid of event MON 810 from Monsanto), the transgenic glyphosate-tolerant Roundup Ready variety DKC78-35R (hybrid of event NK603 from Monsanto), and the near-isogenic non-GM hybrid variety CRN 3505 (Monsanto) were planted at the two locations in South Africa: (a) Petit (over three seasons 2004, 2005, and 2006) and (b) Lichtenburg (2004). Non-GM, Bt, and Roundup Ready maize were planted in triplicate within a randomized block design (3 × 3 blocks). The total size of the field trials was one hectare (ha) (1111 m^2 each block). The maize plants were grown under a high-input system. Plants were fertilized preplanting with 300 kg/ha nitrogen, phosphorus and potassium [4:3:4 (33)], topdressing 300 kg/ha calcium ammonium nitrate (CAN 28), and treated with herbicide 1.8 L/ha Guardian + 200 mL/ha Sumi Alpha. Two months after planting, the plants were treated again with herbicide (2.2 L/ha A-maizing + 1 L/ha Harness + 220 mL/ha alphacypermytrin). Three months after planting, the material was treated with pesticide (750 mL/ha Endosulfan) against stalkborer. The total average yields were 7.8 ton/ha for the non-GM maize line CRN 3505, 8.5 ton/ha for the Bt hybrid variety DKC78-15B, and 8.0 ton/ha for the glyphosate-tolerant Roundup Ready variety DKC78-35R.

The maize was harvested 8 months after planting after being left to dry in the field (12.5% moisture). For the field trial performed at the growing location Petit in 2005, three biological replicate samples (1 kg of maize kernels each) were harvested, and each was analyzed in triplicate (laboratory replicate samples). For all other field trials, one field sample was analyzed in triplicate (laboratory replicates).

German Field Trials. The transgenic Bt hybrid variety TXP 138-F (hybrid of event MON 810 from Monsanto) and its isogenic counterpart DKC3420 (Monsanto) were grown in 2004 at the two locations Neuhof and Pfaffenhofen in Bavaria, Germany. Bt maize and its isogenic counterpart were grown side-by-side in two field plots (each 1575 m^2). Before planting, the soil was fertilized with 140 kg/ha potassium oxide. Plants were fertilized with 84 kg/ha phosphorus pentoxide and 60 kg/ha nitrogen. Ten weeks after planting, the plants were again fertilized with 83 kg/ha nitrogen (deployed as calcium ammonium nitrate). Eight weeks after planting, the plants were treated with herbicide (750 mL of Certrol B, 750 mL of Click, and 30 g Titus/ha). At harvest, four replicate samples (each of 1 kg each) were collected at random at the Neuhof site and three at Pfaffenhofen for the metabolite profiling investigation. All samples were analyzed in triplicate.

Sample Preparation. Maize samples collected from the German sites were subjected to air-drying (30−40 °C) for 3 days after

harvesting. Maize from South Africa was harvested as a dried material. For each field replicate, a subsample of 40 g of maize kernels (10−15% moisture) was frozen in liquid nitrogen and ground with a cyclone mill (Cyclotec, Foss, Germany) equipped with a 500 μm sieve. The flour was freeze-dried (Alpha 1−4 LSC, Christ, Germany) for 48 h. The moisture content of the resulting material (<2%) was determined as loss of weight by drying at 105 °C for 3 h. Freeze-dried flour samples were stored at −18 °C until analysis. The maize samples were coded before analysis; materials from the various field trials were analyzed in a sequential manner.

Metabolite Profiling. *Sample Extraction.* Four hundred milligrams of freeze-dried maize flour was weighed into a 3 mL cartridge (Merck, Darmstadt, Germany), which was sealed with PTFE frits at the bottom and top of the flour layer. The cartridge was connected to a vacuum manifold (Supelco, Taufkirchen, Germany). For disintegration of the matrix, the maize flour was presoaked in 200 μL of methanol for 20 min at ambient temperature with vents of the manifold closed. The methanol was removed by application of vacuum (20−30 mbar max) on the top of the cartridge for 30 min. Lipids were eluted with 4 mL of dichloromethane into 11 mL vials (lipid extract) by gravity flow. Residual dichloromethane was removed from the flour by application of a vacuum on the bottom of the cartridge. Polar compounds were eluted with a total of 10 mL of methanol/water (80 + 20, v + v) within 40 min into 11 mL vials by application of a weak vacuum at the bottom.

Preparation of Standard Solutions. Reference compounds were obtained from Merck KgaA, Fluka (Buchs, Switzerland), Riedel de Haën (Seelze, Germany), and Oxeno (Marl, Germany). Retention time standard mix I: Solutions of undecane (1.5 mL, 1 mg/mL), hexadecane (2.5 mL, 1 mg/mL), tetracosane (4 mL, 1 mg/mL), and triacontane (4 mL, 1 mg/mL) in *n*-hexane were added to 10 mg of octatriacontane. Retention time standard mix II: 1.5 mL of *n*-hexane and solutions of hexadecane (2.5 mL, 1 mg/mL), tetracosane (4 mL, 1 mg/mL), and triacontane (4 mL, 1 mg/mL) in *n*-hexane were added to 10 mg of octatriacontane. Hydrocarbons were purchased from Fluka. Alanine was used as a retention time standard in place of undecane for fraction IV. Internal standard solution for fraction I: Identical to retention time standard mix I. Tetracosane was used as an internal standard for quantification of major lipids. Internal standard solution for fraction II: 6 mg of 5α-cholestan-3β-ol was dissolved in 10 mL of dichloromethane. Internal standard solution for fraction III: 40 mg of phenyl-β-D-glucopyranoside was dissolved in 25 mL of distilled water. Internal standard solution for fraction IV: 20 mg of *p*-chloro-L-phenylalanine was dissolved in 25 mL of distilled water.

Fractionation and Analysis of Lipids. One hundred microliters of internal standard solution for fraction I and 100 μL of internal standard solution for fraction II were added to the lipid extract. The solution was evaporated in 4 mL vials to dryness by rotary evaporation (ACTEVap Evaporator, Activotec, Cambridge, United Kingdom). Residual solvents were removed by application of nitrogen. The lipids were redissolved in 500 μL of dry methyl *tert*-butyl ether and 250 μL of dry methanol, and 50 μL of sodium methylate and 5.4 M in methanol were added. After reaction for 90 min at room temperature in the dark, 1 mL of dichloromethane and 2 mL of aqueous 0.35 M HCl were added. The solution was shaken vigorously, and the upper phase was discarded. After re-extraction of the lower phase containing the transmethylated lipids with another 2 mL of aqueous 0.35 M HCl, the solution was evaporated to dryness by rotary evaporation. The dry transmethylated lipid extract was subfractionated by solid-phase extraction (SPE). After 200−300 mg of sodium sulfate was placed on top of the cartridge, one column volume (CV, 2.5 mL) of *n*-hexane was used for conditioning the SPE column. The *n*-hexane was removed by application of weak vacuum on the bottom. Trans-methylated lipids were redissolved in 250 μL of dichloromethane and transferred to the SPE cartridge. The methyl ester fraction (fraction I) was eluted with 3 × 2 mL of *n*-hexane and MTBE (100:2, v + v). The eluate was evaporated to dryness by rotary evaporation (160 mbar min) and redissolved in 300 μL of *n*-hexane and transferred into a TPX plastic autosampler vial with an integrated 0.2 mL glass microinsert and closed

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using a 6 mm silicone/PTFE red screw cap. One microliter was injected into GC-MS. The minor polar lipid fraction (fraction II) was eluted with 3 × 2 mL of *n*-hexane and MTBE (70:30, v + v). After the addition of 100 μL of retention time standard mix I, the eluate was evaporated to dryness by rotary evaporation (160 mbar min). Residual solvents were removed by application of nitrogen. Fraction II was redissolved in 250 μL of dry pyridine and 50 μL of *N*-methyl-*N*-trimethylsilyl-trifluoracetamide. After it was flushed with argon, the vial was tightly sealed with PTFE sealings and silylated for 15 min at 70 °C. The sample was then transferred to a TPX plastic autosampler vial with an integrated 0.2 mL glass microinsert and closed using a 6 mm silicone/PTFE red screw cap; 1 μL was injected into the GC-MS.

Fractionation of Polar Extract. One hundred fifty microliters of internal standard solution for fraction III and 150 μL of internal standard solution for fraction IV were added to the polar extract. One milliliter of this solution was concentrated in 4 mL vials by rotary evaporation and dried over phosphorus pentoxide. After it was redissolved in 200 μL of dry pyridine and 100 μL of dry trimethyl-silylimidazole was added, the sample was silylated for 20 min at 70 °C in a tightly sealed vial. For differential hydrolysis of the silylated derivatives, 200 μL of *n*-hexane and 400 μL of water were added. After slightly shaking it at room temperature and subsequent phase separation (5 min), 150 μL of the upper phase (fraction III) was transferred into a TPX plastic autosampler vial, and 75 μL of retention time standard mix I was added prior to closing with a 6 mm silicone/PTFE red screw cap. One microliter was injected into the GC-MS. Two milliliters of polar extract was concentrated by rotary evaporation and dried over phosphorus pentoxide. After it was redissolved in 250 μL of dry hydroxylammoniumchloride, the sample was oximated for 30 min at 70 °C. One hundred microliters of MSTFA was added, and after they were flushed with argon, the tightly sealed vials were allowed to stand for 20 min at 70 °C. Five hundred microliters of *n*-hexane and 300 μL of water were added, and after vortexing and phase separation, the upper phase was removed, and the lower phase was re-extraced with 2 × 500 μL of *n*-hexane. The lower phase, containing acids, amino acids, and amines (fraction IV), was concentrated to dryness by rotary evaporation and dried over phosphorus pentoxide. One hundred microliters of retention time standard mix II was added, and the solvent was removed by application of nitrogen. The dry extract was redissolved in 250 μL of dry acetonitrile, and 50 μL of MSTFA was added. After it was flushed with argon, the sample was resilylated for 60 min at 70 °C; 1 μL was injected into the GC-MS.

GC Analysis. The GC conditions were as described previously.[25] GC was performed on a Finnigan TraceGC Ultra (Thermo Electron Corp., Austin, TX) with split/splitless injector combined with a Finnigan Trace DSQ mass spectrometer (Thermo Electron Corp., Austin, TX) with an electron ionization (EI) ion source. The column used was a factorFOUR VF-1 ms, 60 m × 0.32 mm internal diameter (i.d.), coated with a 0.25 μm film of 100% polydimethylsiloxane (Varian, Darmstadt, Germany). Injection was performed in split mode (split flow, 15 mL/min) at an injection temperature of 280 °C. Helium as the carrier gas was used at a constant flow of 1 mL/min. The column temperature was programmed from 100 to 320 °C (10 min hold) at a 4 °C/min. The MS interface temperature was set to 320 °C. After a solvent delay of 6 min, full scan mass spectra were recorded within a scan range of 40−700 mu at an electron energy of 70 eV and a source temperature of 250 °C. The identification of maize constituents was based on comparison of retention times and mass spectra with those of reference compounds.

Statistical Analysis. Retention time matching of GC data was performed by use of Chrom*pare*, a self-tailored MS Excel tool (www.chrompare.com). The tool is optimized for comparison of chromatographic data, including automated retention time adjustment according to retention time standards. Metabolites (identified and unidentified) were standardized and quantified by relative peak height levels according to the respective internal standard. Peaks below a threshold level of 2% relative peak height in fractions I and II and 3% relative peak height in fractions III and IV were excluded from comparisons. Trace constituents for which the confidence intervals ($p < 0.05$) were higher than their mean levels were also not included for comparison.

The principal component analyses (PCAs) were performed on the basis of values from triplicate analyses by means of XLStat 2008 (Addinsoft, France). Environment and genotype means were compared by analysis of variance (ANOVA) using plot/triplicate as the error model. All analyses of variance were performed using GenStat 14.1 (VSN International Ltd., United Kingdom).

■ RESULTS

Metabolite profiling of GM maize (Bt hybrid variety TXP 138-F grown in Germany; Bt hybrid variety DKC78-15B and glyphosate-tolerant Roundup Ready variety DKC78-35R grown in South Africa) and the corresponding isogenic lines was performed according to a previously described extraction scheme resulting in four fractions containing major lipids (fraction I), minor lipids, for example, free fatty acids and sterols (fraction II), sugars and sugar alcohols (fraction III) and acids, and amino acids and amines (fraction IV).[25] GC-MS analysis enabled the coverage of a broad spectrum of primary metabolites (e.g., fatty acid methyl esters) as well as of nutritionally relevant minor maize constituents (e.g., tocopherols) to be assessed by means of multivariate (PCA) and univariate (ANOVA) statistical analyses. On average, for the growing locations in Germany and South Africa, 146 and 120 peaks, respectively, were included for the data assessment of GM and isogenic maize. An overview on the maize constituents covered by the employed GC-MS metabolite profiling is given by Röhlig et al.[23]

Bt Hybrid Variety TXP 138-F. *Multivariate Analysis.* GC-MS metabolite profiling data of Bt hybrid variety TXP 138-F and its near-isogenic counterpart, grown under conventional farming practice at the two locations Neuhof and Pfaffenhofen in Germany, were subjected to multivariate PCA (Figure 1). PCA from the combined nonpolar and polar metabolite fractions I−IV revealed a distinct separation of the two growing locations on the first principal component representing 34% of the total variation (Figure 1A). In contrast, for both locations, no clear separation was observed between the Bt maize and the non-GM comparator. The employed fractionation procedure allowed a closer analysis of the metabolites responsible for the overall observed differentiation. The results obtained by PCA for the individual fractions I, II, III, and IV demonstrate unequal contributions of the maize metabolites from different chemical classes (Figure 1B−E). PCAs and corresponding PCA factor loading scores (Figure 2) clearly revealed compounds from the two polar fractions containing sugars and sugar alcohols and acids, amino acids, and amines as the major drivers of variation between the two locations Neuhof and Pfaffenhofen (Figures 1 and 2). However, none of the single fractions containing major and minor maize constituents showed a distinct separation of Bt maize from the near-isogenic line in PC1. Only for fraction IV is there a small indication of a separation along PC2 between Bt maize and the near-isogenic line for the samples grown in Neuhof (Figure 1E). PCA score plots considering further principal components (e.g., PC1 and PC3, PC2, and PC3) did also not result in more pronounced differentiations according to the genetic modification. When distinguishing between analytical and field replicates (Figure 1F), it becomes obvious that the variation between Bt and isogenic maize is not higher than that between field replicates.

Univariate Analysis. The assessment of PCA factor loadings (Figure 2) is a suitable approach to determine the major metabolites responsible for the clustering seen for the two growing locations (Figure 1B−E). However, these loadings may reflect
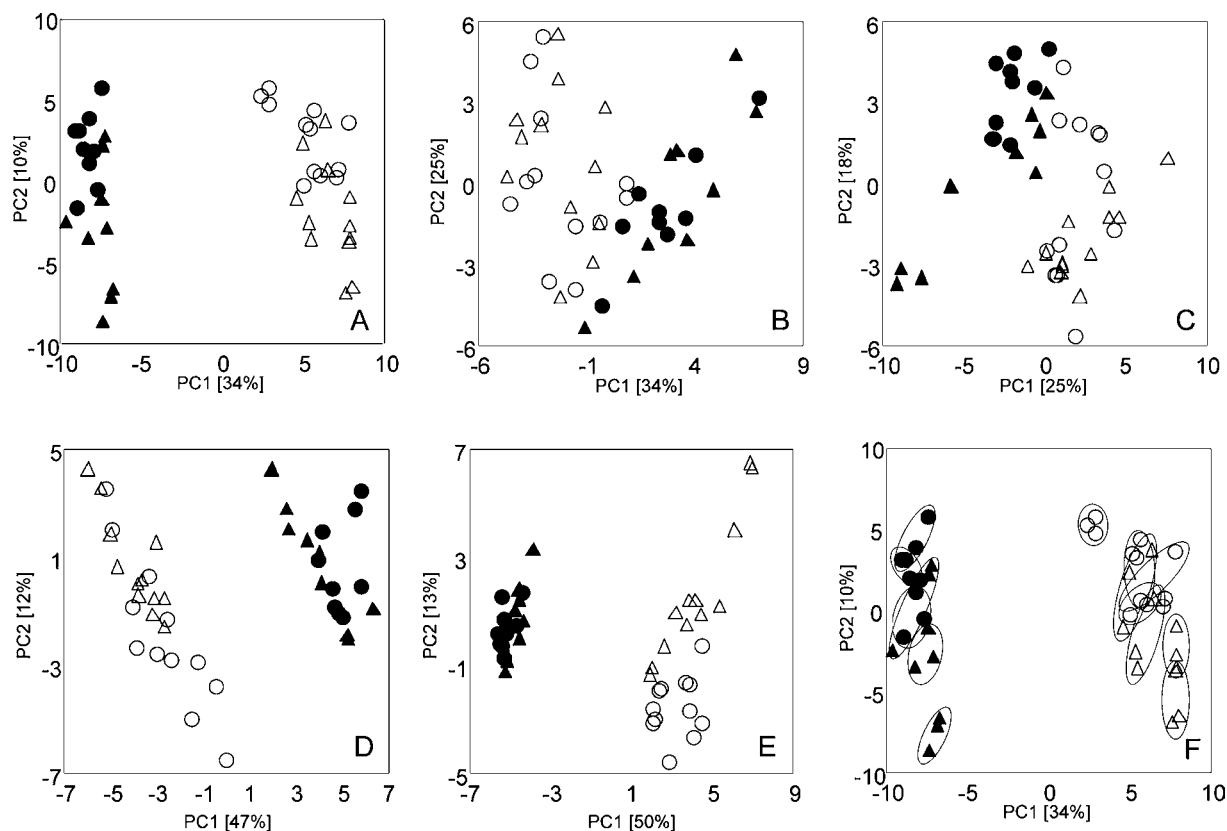
Figure 1. Principal component analysis of GC-MS metabolite profiling data from combined fractions I–IV (A and F) and of fractions I (B), II (C), III (D), and IV (E) of Bt-maize (▲, △) and its isogenic counterpart (●, ○) grown at Pfaffenhofen (▲, ●) and Neuhof (△, ○). Three and four field replicates at Pfaffenhofen and Neuhof, respectively, were analyzed in triplicate. The circles in plot F indicate the data from triplicate analysis of field replicates.

metabolic changes due to both the genetic modification and the environmental impact (different farming locations).

To solely assess the impact of the factor genotype, that is, GM versus non-GM, metabolite profiling data of Bt maize and the isogenic line were subjected to an univariate ANOVA. A total of 146 peaks (peak heights standardized related to the internal standard) were considered for the comparison of TXP 138-F and the isogenic line DKC3420. No additional or missing peaks were found for the Bt maize as compared to the isogenic line grown at the two locations. The raw data underlying the performed ANOVA are provided in the Supporting Information, Table 1. The ANOVA resulted in the detection of six metabolites exhibiting statistically significant differences in peak heights ($p <$ 0.01) between the Bt maize and the isogenic counterpart; this accounts for 4% of the total number of covered peaks. Mean responses relative to the internal standard of the respective fraction are shown in Table 1. As compared to the factor genotype (GM vs non-GM), an ANOVA considering the factor environment, that is, the two growing locations, revealed a higher number of significant differences between maize grown at Pfaffenhofen and Neuhof (Table 2). For 62 metabolites, statistically significant differences in peak heights between the two growing locations were found, representing 42% of the total covered peaks.

Bt Hybrid Variety DKC78-15B and Roundup Ready Variety DKC78-35R. *Multivariate Analysis.* For the assessment of the overall metabolic variation in GM and non-GM maize lines, a PCA of data from Bt maize, Roundup Ready (RR) maize, and the near-isogenic maize grown 2004 at two locations in South Africa was conducted (Figure 3A). The first

two principal components, accounting for 41% of the total metabolic variation, revealed a clustering of GM and isogenic maize lines. At the growing location Petit, Bt maize, Roundup Ready maize, and the respective isogenic counterpart were grown side-by-side in three consecutive seasons (2004−2006). For the growing season 2005, three field replicates at Petit were analyzed in triplicate. A PCA of the metabolite profiling data is shown in Figure 3B. The PCA revealed no clear separation of GM vs non-GM maize grown over the three seasons, indicating the pronounced environmental impact caused by the different growing seasons and, for the maize grown in 2005, the three field replicates.

Univariate Analysis. A total of 120 peaks obtained from the GC metabolite profiling of DKC78-15B (Bt), DKC78-35R (RR), and the near-isogenic, non-GM hybrid variety CRN 3505 were considered for the ANOVA. The raw data underlying the performed ANOVA are provided in the Supporting Information, Table 2. The ANOVA resulted in the detection of four metabolites exhibiting statistically significant differences in peak heights ($p <$ 0.01) between the isogenic and the GM maize lines; this accounts for 3% of the total number of covered peaks. Mean responses relative to the internal standard of the respective fraction are shown in Table 3. An ANOVA considering the factor environment, that is, the two growing locations/seasons, revealed 12 metabolites, representing 10% of the total covered peaks, to be statistically significantly different in peak heights (Table 4). This number of differences is higher than those determined for the factor genotype but lower than
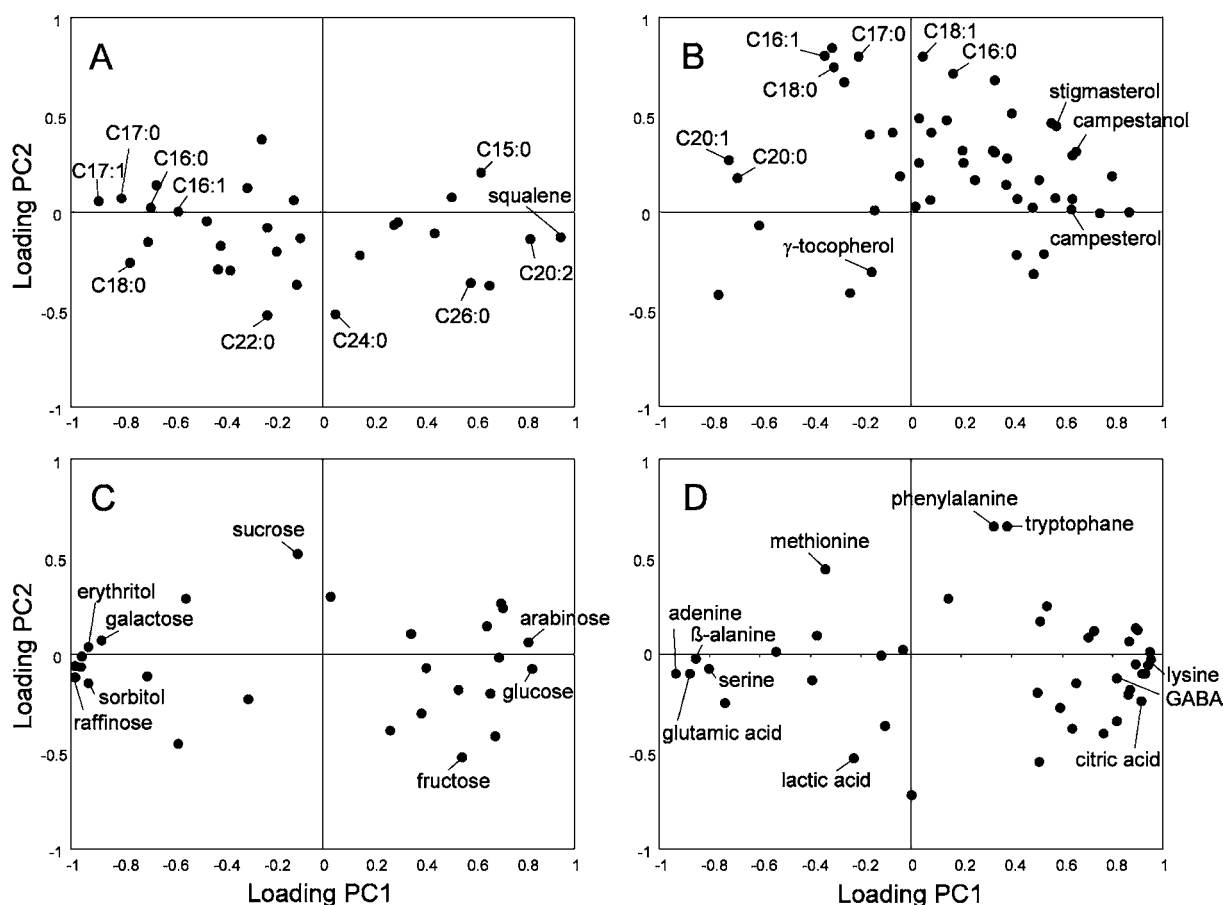
Figure 2. PCA factor loading scores of GC-MS metabolite profiling data from fractions I (A), II (B), III (C), and IV (D) of Bt-maize and its isogenic counterpart grown at Pfaffenhofen and Neuhof.

Table 1. Mean Responses of Peaks Exhibiting Significantly Different Heights ($p < 0.01$) for Non-GM (ISO) and GM (Bt) Maize Grown at the Two Locations Pfaffenhofen and Neuhof (Germany) Based on an ANOVA for the Factor Genotype (Non-GM vs GM)

	response[a]	
compd	ISO	Bt
palmitoleic acid (FFA)[b]	0.046	0.035
NI[c]	0.608	0.488
citric acid	1.545	2.124
phenylalanine	0.124	0.091
tryptophan	0.049	0.021
NI[d]	0.323	0.128

[a]Peak height relative to the internal standard of the respective fraction. [b]FFA, free fatty acid. [c]Not identified; compound present in fraction II. [d]Not identified; compound present in fraction IV.

the number observed for the factor environment at the two German growing locations.

■ DISCUSSION

Considering the total number of peaks detected by GC-MS, univariate data analysis revealed only few differences in the peak heights (accounting for 4 and 3%, respectively) between GM and non-GM maize grown in Germany and South Africa. In accordance with the present study, targeted compositional analyses (taking into account on average 51 components) of

GM drought-tolerant, insect-resistant, and herbicide-tolerant maize grown over several seasons at different locations in Europe, South America, and the United States revealed significant differences between GM vs non-GM maize ranging from 3.4 to 19.5% for single field trials.[22] However, because of a lack of consistently observed differences across several growing locations/seasons and the fact that the levels of compounds reported for GM maize were within the ranges reported for existing conventional-bred maize (e.g., the ILSI Crop Composition Database),[28] the authors of previously published targeted studies on Bt maize and Roundup Ready maize[29−32] claimed compositional equivalence of the investigated GM material as compared to the conventional comparators. On the basis of that data, it was concluded that "compositional differences between GM varieties and their conventional comparators were encompassed within the natural variability of the conventional crop and that the composition of GM and conventional crops cannot be disaggregated".[22]

Multivariate and univariate data analyses demonstrated a pronounced impact of the factor environment on the metabolite profiles of maize grown in Germany and South Africa. Several factors can affect the natural variability of crop metabolites. Prominent contributors to the metabolite phenotype are genetic backgrounds (e.g., different cultivars), breeding strategies (e.g., mutation breeding), environmental conditions (e.g., growing location, season), and farming practices (e.g., organic farming). Various nontargeted analyses have been conducted to investigate the impact of such factors on the crop metabolite profiles.[33] Application of a GC-MS metabolite profiling approach similar to

Table 2. Mean Responses of Peaks Exhibiting Significantly Different Heights ($p < 0.01$) for Non-GM and GM Maize Grown at the Two Locations Pfaffenhofen (PAF) and Neuhof (NH) (Germany) Based on an ANOVA for the Factor Environment (PAF vs NH)

compd	response[a] PAF	response[a] NH	compd	response[a] PAF	response[a] NH
		fraction I			
C15:0 FAME[b]	0.026	0.032	C19:1 FAME	0.057	0.049
C17:0 FAME	0.249	0.214	C17:2 FAME	0.067	0.059
C18:0 FAME	6.023	5.212	C20:2 FAME	0.085	0.102
C17:1 FAME	0.099	0.073	squalene	0.294	0.491
		fraction II			
C23:0 (FFA)[c]	0.033	0.038	NI	0.025	0.041
campesterol	0.815	0.868	NI	0.233	0.153
stigmasterol	0.352	0.380	NI	0.099	0.122
campestanol	0.216	0.237	NI	0.041	0.049
24-MCA[d]	0.024	0.036	NI	0.044	0.058
methyl ferulate	0.136	0.210	NI	0.023	0.032
NI[e]	0.018	0.003			
		fraction III			
erytritol	0.114	0.065	NI	0.143	0.326
sorbitol	2.078	0.945	NI	0.011	0.038
arabinose	0.000	0.020	NI	0.004	0.053
arabinose	0.037	0.058	NI	0.018	0.082
galactose	0.426	0.260	NI	0.046	0.000
glucose	6.213	8.683	NI	0.117	0.000
raffinose	1.279	0.165	NI	0.081	0.065
NI	0.000	0.037	NI	0.076	0.002
		fraction IV			
glycine	0.211	0.460	pyrimidine	0.038	0.007
β-alanine	0.369	0.132	cis-aconitic acid	0.012	0.058
valine	0.368	0.456	citric acid	0.889	2.544
proline	3.836	5.419	glyceric acid	0.037	0.165
serine	0.901	0.552	threonic acid	0.046	0.116
threonine	0.334	0.274	p-cumaric acid	0.000	0.052
glutamic acid	1.301	0.565	NI	0.000	0.035
pyroglutamic acid	0.591	0.910	NI	0.063	0.347
lysine	0.176	0.572	NI	0.420	1.691
tyrosine	0.271	0.414	NI	0.000	0.048
GABA	0.757	1.285	NI	0.317	0.892
citrullin	0.000	0.072	NI	0.027	0.070
adenine	0.088	0.037			

[a]Peak height relative to the internal standard of the respective fraction. [b]FAME, fatty acid methyl ester. [c]FFA, free fatty acid. [d]24-MCA, 24-methylene cycloartanol. [e]Not identified.

the one employed in the current study revealed 15−25% significant differences between four maize cultivars grown at one location over three consecutive years.[25] A comparable number of significant differences (11−30%) were also observed when comparing wild-type maize and low phytic acid mutants generated through mutation breeding.[34] In addition to targeted analyses,[5] nontargeted metabolite profiling of maize grown at different locations over several years confirmed the pronounced environmental impact on the metabolic profiles shown in the present study (Tables 2 and 4). Up to 30 and 41% significant differences in peak heights, respectively, were found for maize grown at different locations and seasons.[25]

In addition to the strong influence of different growing locations on metabolite variation, differences between field
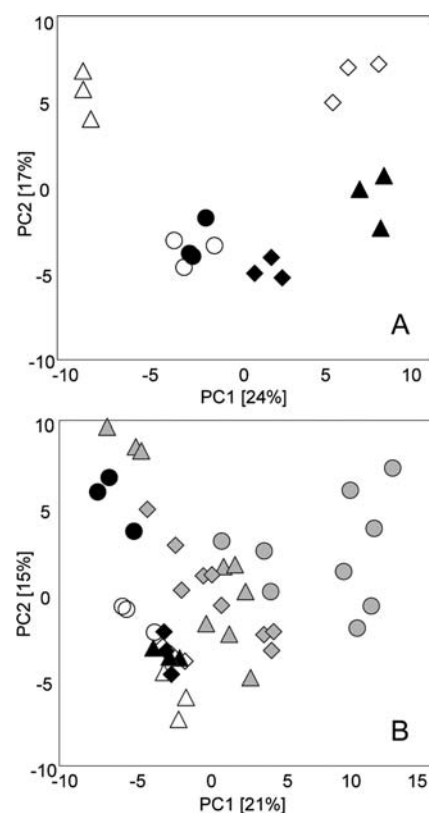

Figure 3. Principal component analysis of GC-MS metabolite profiling data (triplicate analysis of combined fractions I−IV) of Bt maize (△, ▲), Roundup Ready maize (◇, ◆), and the near-isogenic counterpart (○, ●) grown at the locations Lichtenburg (white symbols) and Petit (black symbols) in 2004 (A) and at Petit in 2004 (○, △, ◇), 2005 (gray circle, gray triangle, gray diamond) and 2006 (●, ▲, ◆) (B). For Petit 2005, three field replicates were analyzed in triplicate.

Table 3. Mean Responses of Peaks Exhibiting Significantly Different Heights ($p < 0.01$) for Non-GM (ISO) and GM (Bt, RR) Maize Grown at the South African Field Trials Based on an ANOVA for the Factor Genotype (ISO vs GM)

compd	response[a] ISO	Bt	RR
γ-tocopherol	0.166	0.155	0.053
NI[b]	0.017	0.032	0.004
NI[b]	0.039	0.047	0.032
myo-inositol	0.254	0.232	0.163

[a]Peak height relative to the internal standard of the respective fraction. [b]Not identified; compound present in fraction II.

replicates for maize grown at one location were also observed in the present study. A comparable metabolite profiling study also revealed also differences between maize genotypes harvested from three plots at one location.[25] This is likely explained by differences in the local microclimate and soil conditions.

A recently published metabolite profiling-based study demonstrated the potential impact of an organic farming management practice on maize metabolite profiles.[26] Although the impact of crop management practice on the metabolite phenotype was shown to be minor as compared to genetic background and environment, such effects will contribute to the natural variability of maize metabolite profiles.

Table 4. Mean Responses of Peaks Exhibiting Significantly Different Heights ($p < 0.01$) for Non-GM and GM Maize Grown at the South African Field Trials Based on an ANOVA for the Factor Environment (Comparison of Field Trials)

compd	response[a]			
	Lichtenburg 2004	Petit 2004	Petit 2005	Petit 2006
C15:1 FAME[b]	0.025	0.025	0.012	0.023
sitostanol	1.045	1.064	0.913	1.164
NI[c]	0.043	0.045	0.034	0.047
erythritoll	0.072	0.042	0.099	0.042
glucose	1.284	1.292	1.893	0.984
NI[d]	0.014	0.000	0.063	0.000
alanine	0.208	0.194	0.428	0.197
glycine	0.094	0.091	0.133	0.083
glutamic acid	0.304	0.300	0.104	0.292
pyroglutamic acid	0.298	0.220	0.160	0.268
GABA	0.089	0.055	0.463	0.031
ethanolamine	0.021	0.006	0.052	0.000

[a]Peak height relative to the internal standard of the respective fraction. [b]FAME, fatty acid methyl ester. [c]Not identified; compound present in fraction II. [d]Not identified; compound present in fraction III.

In conclusion, the applied metabolite profiling approach allowed a comprehensive assessment of the metabolite profiles from GM Bt maize and Roundup Ready maize as compared to the non-GM near-isogenic comparators. On the basis of the number of significant differences, the data obtained from Bt maize and Roundup Ready maize grown in South Africa and Germany indicated that environmental influences on the metabolite profiles of the investigated maize genotypes were far more pronounced than the effect of the genetic modification.

The authors are aware that the maize samples investigated, although grown at different locations and seasons, were not fully replicated, as suggested, for example, by the guidelines of the EFSA Panel of Genetically Modified Organisms (GMO) on the statistical requirements for the safety assessment of GM crops.[21] The guidelines indicate that field experiments have to be conducted on the basis of randomized block designs repeated at multiple sites over many years to capture adequately the full extent of the environmental impacts on the crop metabolic phenotype. This is especially important as metabolic alterations in crops due to a genetic modification might be differently expressed in the various environments.

In addition, the employed GC-based methodology is of course not unbiased because it is not applicable to all chemical classes of crop compounds, for example, because of thermal instability or nonvolatility. Nevertheless, the employed metabolite profiling approach in combination with the applied multivariate and univariate data assessment was shown to be a useful tool for the comparative assessment of GM crops in the light of natural variability. In addition, metabolite profiling has the potential to complement existing targeted analytical methods for the analysis of the inherent natural crop metabolic variability caused by various environmental factors. However, it should be kept in mind that for a comprehensive safety assessment not only the number of statistically significant differences but also their toxicological/nutritional implications have to be taken into account.

■ ASSOCIATED CONTENT

⑤ Supporting Information

German and South African maize data and summary ANOVA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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