JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 6041–6049 6041
DOI:10.1021/jf900811u

NMR Metabolic Profiling of Transgenic Maize with the *Cry1A(b)* Gene

Fabiana Piccioni,[†] Donatella Capitani,[‡] Lello Zolla,[†] and Luisa Mannina*[,‡,§]

[†]Dipartimento di Scienze Ambientali, Università degli Studi della Tuscia, I-01100 Viterbo, Italy, [‡]Istituto di Metodologie Chimiche, CNR, I-00015 Monterotondo Staz. Roma, Italy, and [§]Dipartimento di STAAM, Università degli Studi del Molise, I-86100 Campobasso, Italy

The metabolic profiles of seeds from the transgenic maize variety 33P67 and of the corresponding traditional variety were investigated using one- and two-dimensional NMR techniques. The transgenic variety carries a functional *Cry1A(b)* gene, which confers to the plant the ability to produce *Bt* insect toxin. About 40 water-soluble metabolites in the maize seed extracts were identified, providing a more complete ^{1}H and ^{13}C NMR assignment with respect to the assignment reported in the literature. In particular ethanol, lactic acid, citric acid, lysine, arginine, glycine-betaine, raffinose, trehalose, α-galactose, and adenine were identified for the first time in the ^{1}H NMR spectrum of maize seeds extracts. The ^{1}H spectra of transgenic and nontransgenic seed maize samples turned out to be conservative, showing the same signals and therefore the same metabolites. However, a higher concentration of ethanol, citric acid, glycine-betaine, trehalose, as well as of another compound not yet completely identified, was observed in the transgenic extracts than in nontransgenic samples. So, it was possible to discriminate between transgenic and nontransgenic metabolic profilings through the use of an appropriate statistical analysis.

KEYWORDS: Transgenic maize seed; metabolic profiling; NMR; statistical analysis.

INTRODUCTION

The introduction of exogenous DNA sequences into the plant genome to confer new properties and abilities to the plant is an active research field. The transcriptional products of the introduced gene(s), and possibly the modified biochemical pathways based on them, may interact with the regulation of other genes or biochemical pathways. A major concern is the possible occurrence of unintended effects caused, for instance, by the site of transgene integration (e.g., interruption of important open reading frames or regulatory sequences), which could result in a modified metabolism (*1*, *2*).

The safety testing of genetically modified organisms (GMOs) is a high priority for regulatory authorities, and there is a need for techniques capable of detecting any unintended effect following a genetic modification (*3*).

Different complementary approaches can be used to investigate transgenic systems. In particular, genomic, proteomic, and metabolomic profiles are useful as complementary tools for the safety assessment of GM crops (*2*), each technique giving a specific contribution to the overall understanding of the system. The NMR-based approach, used for the identification and quantification of intracellular and extracellular metabolites, gives a direct metabolic fingerprint of the system under investigation (*4*). It allows the simultaneous detection of many compounds in amounts comparable to those detected using standard biochemical assays (*5*) and it does not require extraction procedures specific to each class of compounds. Using NMR, in vivo and in vitro, metabolic alterations have been discovered in biological systems that are of primary importance (*6*, *7*) and these include transgenic foodstuffs such as transgenic tomatoes (*3*, *8*) and transgenic lettuce (*9*). NMR approach has been also used together with a multivariate statistical analysis to study La73-*Bt* maize seeds (*10*−*12*).

The GM maize seed metabolic profile is of particular interest because maize products are an important source of nutrients and, therefore, an essential part of the human diet (both through direct consumption and animal feeds). Commercial GM and non-GM maize seeds have already been studied using a proteomic-based methodology as well as a metabolomic-based approach to identify intended and unintended changes caused by genetic engineering (*13*) and to identify potential marker metabolites using mass spectrometry (*14*).

In this paper, the aqueous extracts of seeds from the transgenic maize variety 33P67 together with the corresponding isogenic control of the maize line 33P66 were investigated by means of 1D and 2D NMR techniques. The basic aims were to study in details the metabolic profilings of the seed extracts and to investigate their variations associated with genetic modifications in the maize seeds.

MATERIALS AND METHODS

Seed Collection and Plant Growth. Maize seed samples were derived from the transgenic commercial *Bt* hybrid line 33P67 (event MON810 from Pioneer Hi-Bred International, Inc., Johnston, IA) and the respective control isoline 33P66. Maize seeds were grown side-by-side

*To whom correspondence should be addressed. Phone: +39 0874 404648. Fax: +39 06 90672477. E-mail: mannina@unimol.it.

Published on Web 06/22/2009

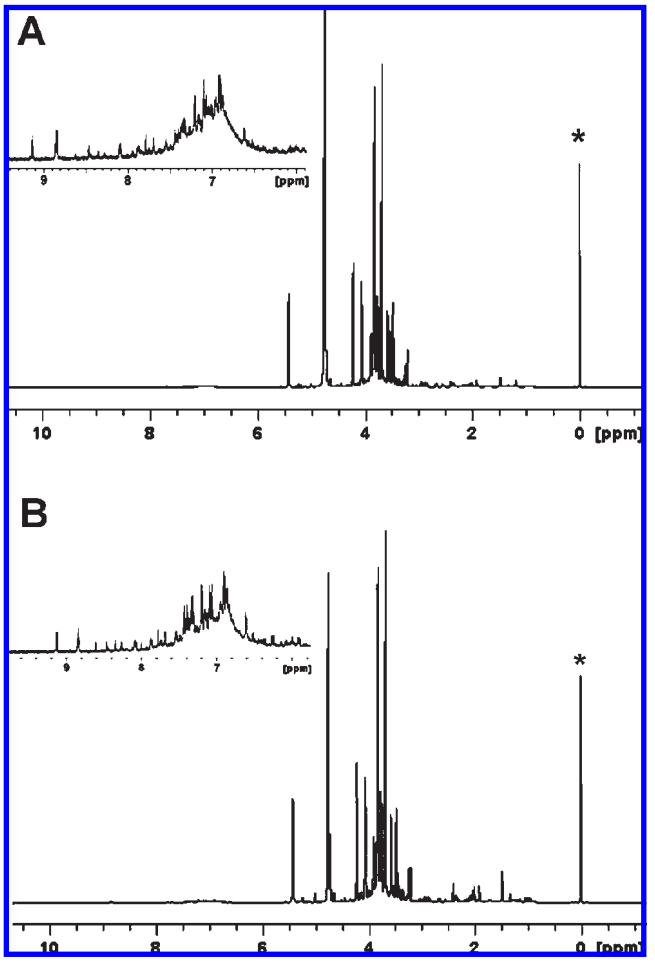

Figure 1. ^{1}H NMR spectrum (600.13 MHz) at 300 K of (**A**) GM maize aqueous extract and (**B**) non-GM maize aqueous extract. The vertical expansion of 5.90−9.60 ppm spectral region is reported as inset. (∗) TSP signal used as internal standard.

in environmentally controlled growth chambers, adjacent but separated to prevent any cross pollination and to obtain control and transgenic lines in the same environmental conditions. Plants were grown at 25 °C with 70% relative humidity and 14 h of light with a light regime of about 100 μmol m^{-2} s^{-1}). The phenotypes of transgenics and control samples were identical. GM and non-GM samples were harvested simultaneously; the ears were harvested after physiological maturity, dried at 30 °C, and stored in sealed plastic bags at 4 °C. Seeds derived from the central portion of a single ear were used for the analysis.

The stability of the genome was guaranteed by the producer company through multiple generations of crossing. Maize seeds were genetically modified using the particle bombardment technique, which involves the random bombardment of cells with metal particles carrying the transgene (*15*).

Extraction Procedure. Each maize seed was weighed (~200 mg) and then frozen in a stainless steel mortar using liquid N_2. It was then pulverized to a fine powder with a pestle previously chilled in liquid N_2. Samples were extracted according to the Bligh−Dyer procedure (*16*), modified to avoid artifacts arising from the breakdown of macromolecular structures (*17*). Pellets were mixed with 4 mL of cold CH_3OH:$CHCl_3$ (2:2) followed by 1 mL of cold H_2O and centrifuged at 10000*g* for 20 min at 4 °C. The resulting upper hydro-alcoholic and lower chloroformic phases were separated. The extraction procedure was performed twice on the pellet in order to obtain a quantitative extraction. After the second extraction, the two hydro-alcoholic phases were pooled and dried under N_2 flow and stored at −80 °C. The same procedure was performed to obtain 10 extracts of transgenic and 10 of control.

^{1}H NMR Analysis. Dry samples were dissolved in a D_2O phosphate buffer (100 mM, pH 7.2) containing trimethylsilylpropionate (TSP, 1 mM) as internal standard.

Table 1. Metabolites Identified in the 600 MHz ^{1}H Spectrum of the Aqueous Extract of Maize 33P67 at pH 7.2 (Phosphate Buffer)[a]

compd	assignment	^{1}H (ppm)	multiplicity	^{13}C (ppm)
	Carbohydrates			
sucrose (Suc)	CH-1 (Glc)	5.42	d	93.20
	CH-2	3.57	dd	72.10
	CH-3	3.77	t	73.60
	CH-4	3.48	t	69.95
	CH-5	3.85		
	CH_2-6	3.83		
	CH_2-1′ (Fru)	3.68	s	62.03
	CH-2′			104.6
	CH-3′	4.22	d	77.59
	CH-4′	4.06		75.00
	CH-5	3.89	m	82.30
	CH_2-6	3.83		62.99
β-glucose (β-Glc)	CH-1	4.64	d	96.37
	CH-2	3.24	t	75.20
	CH-3	3.51	t	76.86
	CH-4	3.40	dd	70.65
	CH-5	3.48	dd	77.05
	CH_2-6,6	3.72, 3.90	dd	61.35
α-glucose (α-Glc)	CH-1	5.24	d	93.10
	CH-2	3.54	dd	72.50
	CH-3	3.73	dd	73.80
	CH-4	3.42	t	70.10
	CH-5	3.84		72.50
	CH_2-6,6	3.77, 3.84		61.70
raffinose	Gal1H	5.00	d	99.30
	Gal2H	3.83		
	Gal3H	3.90		
	Gal4H	4.00		
	Gal5H	3.97		
	Gal6H	3.76		
	G1H (Glc)	5.44		93.20
	G2H	3.57		
	G3H	3.77		73.60
	G4H	3.53		
	G5H	4.06		
	G6H	4.01		
	F1H (Fru)	3.66		
	F2H			
	F3H	4.23		77.20
	F4H	4.06		75.00
	F5H	3.90		82.30
	F6H	3.77		
D-trehalose	C1H	5.20	d	94.30
	C2H	3.65		
	C3H	3.86		
	C4H	3.44		
	C5H, C6H	3.78		
α-galactose (Gal)	C1H	5.27	d	93.13
	C2H	3.77		
β-galactose (Gal)	C1H	4.60	d	97.44
	C2H	3.50		
	Organic Acids			
isobutyric acid (IA)	CH_3	1.14	d	18.00
acetic acid (AA)	α-CH_3	1.91	s	24.10
lactic acid (LA)	β-CH_3	1.33	d	20.60
	α-CH	4.13	q	69.10
malic acid (MA)	α-CH	4.32	dd	
	β-CH	2.68	dd	
	β,β'-CH_2	2.38	dd	
citric acid (CA)	$\alpha\gamma$-CH	2.54	d	45.80
	$\alpha'\gamma'$-CH	2.68	d	45.80

Table 1. Continued

compd	assignment	^{1}H (ppm)	multiplicity	^{13}C (ppm)
pyruvic acid (PA)	β-CH_3	2.37	s	
succinic acid (SA)	α β-CH_2	2.40	s	34.90
fumaric acid (FA)	α,β-CH=CH	6.53	s	
formic acid	HCOOH	8.46	s	
	Amino Acids			
valine (Val)	α-CH	3.61	d	
	β-CH	2.27	m	
	γ-CH_3	0.99	d	
	γ'-CH_3	1.04	d	18.60
isoleucine (Ile)	α-CH	3.65	m	
	β-CH	1.96	m	
	γ-CH_3	1.02	d	
	γ-CH	1.25	m	
	γ'-CH	1.45	m	
	δ-CH_3	0.93	t	
leucine (Leu)	α-CH	3.69	t	
	γ-CH	1.69	m	
	δ-CH_3	0.96	d	22.70
	δ'-CH_3	0.94	d	21.50
lysine (Lys)	α-CH	3.74	t	
	β-CH_2	1.89	m	
	γ-CH_2	1.45	m	
	δ-CH_2	1.69	m	24.80
	ε-CH_2	3.01	t	40.10
alanine (Ala)	α-CH	3.77	q	51.10
	β-CH_3	1.48	d	16.75
threonine (Thr)	α-CH	3.58	d	
	β-CH	4.25	m	
	γ-CH_3	1.32	d	20.00
arginine (Arg)	β-CH_2	1.65; 1.72	m	26.60
	γ-CH_2	1.92	m	30.20
	δ-CH_2	3.23	t	
asparagine (Asn)	α-CH	4.00	dd	
	β-CH	2.86	dd	35.10
	β'-CH	2.95	dd	35.10
aspartate (Asp)	α-CH	3.89	dd	
	β-CH	2.68	dd	37.20
	β'-CH	2.79	dd	
γ-aminobutyrate (GABA)	α-CH_2	2.28	t	35.00
	β-CH_2	1.88	quintet	
	γ-CH_2	3.01	t	40.10
glutamate (Glu)	α-CH	3.75	t	55.20
	β-CH	2.09	dt	27.69
	β'-CH			
	γ-CH_2	2.34	t	34.10
glutamine (Gln)	α-CH	3.77	t	
	β-CH_2	2.15	m	27.13
	γ-CH_2	2.45	m	31.40
proline (Pro)	α-CH	4.14	t	
	β-CH	2.36	m	29.61
	β'-CH	2.08	m	24.30
	γ-CH_2	2.01	m	
	δ-CH	3.40	t	
	δ'-CH	3.33	t	
tyrosine (Tyr)	α-CH	3.93	dd	
	β-CH	3.17	dd	
	β'-CH	3.05	dd	
	C2,6H ring	7.18	d	
	C3,5H ring	6.88	d	116.05
histidine (His)	C2H ring	7.05	s	116.53
	C4H ring	7.78	s	
phenylalanine (Phe)	CH-2,6	7.31	m	
	CH-3,5	7.44	m	
tryptophan (Trp)	C5H ring	7.20	t	
	C6H ring	7.28	t	
	C7H ring	7.54	d	
dimethylamine (DMA)	CH_3	2.73	s	35.97
trimethylamine	CH_3	2.91	s	37.86
	Osmolytes			
Trigonelline	N$-CH_3$	4.05	s	
	HB, HC	8.06		127.92
	HB, HC	8.84	d	71.10
	HA	9.13	s	
glycine-betaine (GB)	N$-CH_3$	3.27	s	54.00
	α-CH_2	3.91	s	66.50
V23 (U)	N$-CH_3$	3.23	s	54.96
	Sugar Alcohols			
V21 (U)	N$-CH_3$	3.11	s	47.00
choline	$N(CH_3)_3^+$	3.21	s	54.70
	α-CH_2			68.30
V24 (U)	N$-CH_3$	3.23	s	54.75
				74.87
V25 (U)	N$-CH_3$	3.25	s	41.20
	Alcohols			
ethanol (EtOH)	CH_3	1.18	t	17.57
	CH	3.66	q	66.70
	Phenols			
V39 (U)		7.16	d	
	1H, H-2	7.10	d	
	1H, H-8	6.24	d	
V46 (U)		7.26	d	
		7.10	d	
		7.34		
AA′XX′		6.88	d	116.52
		7.00		
		7.31		
		6.92	d	115.98
		7.54		
		7.60		
V42 (U)		6.42	d	
		6.60	d	116.00
		7.40		
	Nucleotides			
UDP-glucose	C1H (Glc)	5.56	d	93.50
	C2H	3.56		
	C3H	3.76		
UMP	C1′H ribose	5.99	d	
	C6 ring	8.11	d	127.90
V38 (U)		6.00	d	
V51 adenine		8.27	s	122.70
V54 adenine		8.61	s	
V52 (U)		8.34	s	

[a] ^{1}H and ^{13}C chemical shifts are referred to a 1 mM TSP as internal standard. Abbreviations are reported. Chemical shifts are referred to TSP signal (δ = 0.00 ppm) and to anomeric ^{13}C signal of alanine at 16.75 ppm, respectively; s, singlet; d, doublet; dd, double of doublets; t, triplet; q, quartet; m, multiplet; AA′XX′ corresponding to spin systems unidentified; U, unknown.

^{1}H NMR spectra of maize seed extracts were performed at 300 K on a Bruker Avance ASQ600 spectrometer operating at a proton frequency of 600.13 MHz (B_0 = 14.3 T). One-dimensional spectra were acquired by pulse-acquire sequence using the following parameters: 6000 Hz spectral width, 64 K data points, relaxation time 4 s, acquisition time 4.5 s, 256 transients, and 90° flip angle. Water suppression was obtained by presaturation sequence present in the routine experiments.

Two-dimensional (2D) NMR experiments, that is, ^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HSQC, ^{1}H−^{13}C HMBC, and DOSY (*18*) were performed using the same experimental conditions previously reported (*19*): the delay for the evolution of long-range couplings in ^{1}H−^{13}C HMBC was 80 ms, and the mixing time for ^{1}H−^{1}H TOCSY was 80 ms.

Pulsed field gradient spin echo (PGSE) experiments were performed with a pulsed field gradient unit producing a magnetic field gradient in the *z*-direction with a strength of 55.4 G cm^{-1}. The stimulated echo pulse sequence using bipolar gradients with a longitudinal eddy current delay was used. The strength of the sine-shaped gradient pulse with a duration of 1.4 ms was logarithmically incremented in 32 steps, from 2% up to 95% of the maximum gradient strength, with a diffusion time of 130 ms and a longitudinal eddy current delay of 5 ms.

The NMR data were processed using the TOPSPIN software (Bruker).

The quantification of compounds in the maize extracts was obtained by measuring the intensity of selected signals with respect to the intensity of the trimethylsilylpropionate (TSP) signal at 0.00 ppm normalized to 100.

Statistical Analysis. The statistical elaboration of the NMR data was performed using the software package STATISTICA for Windows (version 6.0; 1997). The intensity of the variables was mean-centered and standardized, i.e., divided by standard deviation before performing the (ANOVA) and principal component analysis (PCA).

The ANOVA allows the variables with the highest discriminating power to be selected according to their *p*-level and *F* values. The *p*-level

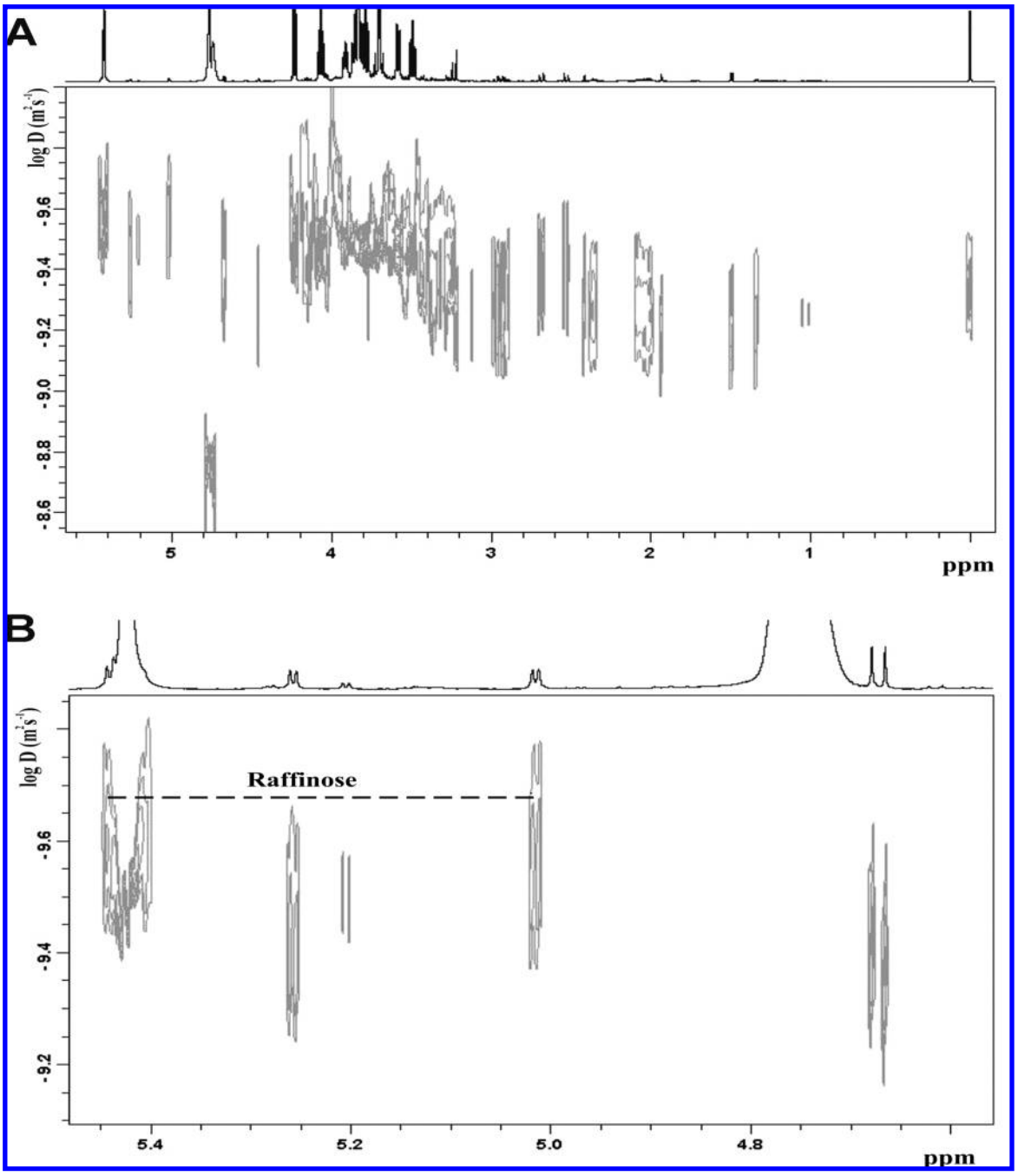

Figure 2. (**A**) Two-dimensional DOSY map of maize seed 33P67 extract in D_2O phosphate buffer at 300 K. The ^{1}H spectrum of the aqueous extract is also reported as horizontal projection. (**B**) Expansion of 4.60−5.45 ppm spectral region in order to highlight the raffinose signal.

represents a decreasing index of the reliability of a result and gives the probability of error involved in accepting a result as valid. A p level < 0.05 (5% probability of error) was treated as a borderline acceptable error level. In particular, the F value is defined as the ratio of the "between-groups variability" and "within-group variability". The larger the ratio, the larger the discriminating power of the corresponding variable.

PCA was performed on the variables selected by ANOVA. PCA results are shown reporting the scores of the principal components and also as a plot of the variable loadings. The percentage of variance for each specific principal component is also reported.

RESULTS AND DISCUSSION

^{1}H NMR Spectral Assignment. In **Figure 1A**, the ^{1}H NMR spectrum of a GM maize seed extract in D_2O phosphate buffer is reported. The assignment of the ^{1}H spectrum was obtained using literature NMR data (*4*, *9*, *20*−*23*) and 2D experiments. Data obtained previously by CE-TOF-MS on the same transgenic line (*14*), see **Table 1**, were also used to confirm some assignments.

Some signals were assigned to the following compounds not previously detected in the ^{1}H NMR spectrum of maize seed extracts (*10*−*12*): ethanol, lactic acid, citric acid, lysine, arginine, glycine-betaine, raffinose, trehalose, α-galactose, and adenine.

Ethanol was identified by means of the diagnostic triplet at 1.18 ppm, which correlates with a signal at 3.66 ppm in the COSY map. The corresponding ^{13}C chemical shifts at 17.57 and 66.70 ppm confirmed the assignment. Lactic acid was identified by the doublet at 1.33 ppm, which correlates with a quartet at 4.13 ppm in the COSY map and by the corresponding carbons at 20.60 and 69.10 ppm. Lysine was identified by its characteristic spin system, in the TOCSY map, consisting of a triplet at 3.01 ppm, three multiplets at 1.69, 1.45, and 1.89 ppm, and a triplet at 3.74 ppm as well as by the corresponding carbons. Arginine shows a spin system consisting of three multiplets at 1.65 and 1.72 ppm, 1.92 ppm and a triplet at 3.23 in the TOCSY map. The corresponding carbons in the ^{1}H−^{13}C map confirmed the structure. Citric acid was identified by the doublet at 2.54 ppm, which correlates with a doublet at 2.68 ppm and by the corresponding carbons, see **Table 1**.

Glycine-betaine, which is an important osmolyte, shows resonances at 3.27 and at 3.91 ppm and the corresponding carbons at 54.00 ppm and 66.50 ppm, respectively.

The ^{1}H spectrum shows also the presence of raffinose, a trisaccharide consisting of galactose, glucose, and fructose units. At 5.00 ppm, the ^{1}H spectrum reveals a diagnostic doublet typical of the α anomeric proton of galactose linked to position 6 of another unit. To establish the nature of the linked unit, a DOSY experiment was performed (*24*). This experiment is a particularly convenient way of displaying the molecular self-diffusion information in a bidimensional array, with the NMR spectrum in one dimension and the self-diffusion coefficient in the other one. DOSY has been successfully used for the analysis of mixtures (*25*) and for the study of intermolecular interactions (*26*, *27*). Here, we report the DOSY map of the hydro-alcholic extract, see **Figure 2A**, **B**. Signals with different diffusion coefficients due to different metabolites and signal with the same diffusion coefficient can be observed. Using DOSY information and 2D experiments, it was possible to identify the raffinose resonances, see **Table 1**. The DOSY map shows that the anomeric proton of galactose has the same diffusion coefficient of a signal at 5.44 ppm partially overlapped to the sucrose doublet. The signal at 5.44 ppm can be attributed to the α glucose linked to both galactose and fructose (*4*). Note that, as aspected, the diffusion coefficient of raffinose, a trisaccharide, is lower than the diffusion coefficient of the glucose, a monosaccharide, and of sucrose, a disaccharide. The presence of raffinose, not previously identified in the seed maize extracts, was also confirmed by the addition of a standard compound in the samples. Raffinose family oligosaccharides have different roles in plants, being used for the transport and storage of carbon and as compatible solutes for protection against abiotic stress (*28*−*30*). They are almost ubiquitous in seeds and have been hypothesized to constitute an important energy source during germination (*31*).

Table 2. ANOVA Applied to 39 Metabolites to Discriminate between GM and Non-GM Maize Seeds Hydro-alcoholic Fractions[a]

	F (Fisher)	p-level	order
V16 CA	3.06157	0.0472	t > c
V25 (U)	4.591874	0.0460	
V17 DMA	4.951564	0.0391	
V41 FA	5.054791	0.0373	
V24 (U)	5.887674	0.0260	
V37 UMP	6.301190	0.0218	
V30 (U)	6.414180	0.0208	
V38 (U)	6.450670	0.0205	
V26 Suc	6.586361	0.0194	
V50 (U)	6.750600	0.0181	
V32 β-Glc	6.811604	0.0177	
V44 Tyr	8.288630	0.0099	
V58 GB	8.777572	0.0083	t > c
V5 EtOH	9.528544	0.0064	t > c
V52 (U)	10.44380	0.0046	
V13 PA	10.65512	0.0043	
V51 adenine	10.80720	0.0041	
V12 Glu	13.63309	0.0016	
V21 (U)	15.45155	0.00098	t > c
V34 α-Glc	19.09433	0.00036	
V39 (U)	20.12644	0.00028	
V48 Trp	20.91203	0.00023	
V59 D-trehalose	22.50214	0.00016	t > c
V54 adenine	24.91015	9.47×10^{-5}	
V14 SA	28.10306	4.86×10^{-5}	
V56 LA	30.01585	3.33×10^{-5}	
V11 GABA	41.36352	4.71×10^{-6}	
V42 (U)	43.15032	3.59×10^{-6}	
V9 Pro	45.77859	2.44×10^{-6}	
V33 raffinose	46.97719	2.06×10^{-6}	
V8 AA	50.72701	1.23×10^{-6}	
V47 Phe	51.50064	1.11×10^{-6}	
V2 Ile	55.04748	7.04×10^{-7}	
V57 Thr	60.13772	3.81×10^{-7}	
V7 Ala	79.52399	5.05×10^{-8}	
V23 (U)	81.11446	4.36×10^{-8}	
V3 Val	90.84033	1.86×10^{-8}	
V1 Leu	102.4051	7.44×10^{-9}	
V4 IA	143.1754	5.29×10^{-10}	

[a] Variables with p-levels less than 0.05 are reported. Abbreviations: see list in **Table 1**, U, unknown; c, control; t, transgenic.

In seed extracts from another maize variety, La73-*Bt* (*10*), the signal at 5.00 ppm has been attributed to melibiose, a disaccharide consisting of galactose and glucose units.

In addition, a signal reported as V21 was not yet fully assigned due to its extremely low concentration. The V21 singlet at 3.11 ppm with corresponding carbon at 47.00 ppm in the HSQC map is due to the methyl signal of the N−CH_3 group of a compound similar to choline.

The ^{1}H spectrum shows also the presence of trehalose, a nonreducing disaccharide sugar composed of two glucose units joined by an α, α-1, 1 linkage. It was assigned by means of the doublet signal at 5.20 ppm with a coupling constant of 3.9 Hz, by the corresponding carbon at 94.30 ppm, and by the spin system identified in the TOCSY map, see **Table 1**.

In **Figure 1A**, the low-field region, from 5.90 to 9.60 ppm, is also reported. This low-field spectral region shows many signals.

Some aromatic compounds such as tyrosine, phenylalanine, tryptophan, adenine, pyrimidine nucleoside, phenolic acids, formic acid, and trigonelline (a product from the metabolism of vitamin B_3) were identified.

The presence of adenine, not previously identified in the ^{1}H spectrum but observed in the mass electropherograms, (*14*) was suggested by the diagnostic singlets at 8.27 ppm with corresponding carbon at 122.7 ppm and the signal at 8.61 ppm.

Several compounds present in extremely low concentrations were not completely assigned, but some structural information can be given. For instance, the presence of a chemical structure similar to the caffeoyl system was suggested by the presence in the TOCSY map of a spin system consisting of a doublet at 7.16 ppm, a doublet at 7.10 ppm, and a doublet at 6.24 ppm (*32*) with a coupling constant of 15.75 Hz. Two AA′XX′ systems with signals at 6.88, 7.00, and 7.31 ppm and 6.92, 7.60, and 7.54 ppm, respectively, matched well with a tyrosine structure. Finally, a group of signals at 6.42, 7.40, and 6.60 ppm in the TOCSY-COSY experiments, with a correlation between 6.60 and 116 ppm in HSQC, were observed.

It is important to underline that the NMR and CE-TOF-MS techniques provide different and complementary information. For example, among the 10 compounds identified for the first time in the ^{1}H NMR spectrum of maize seed extracts, only adenine and arginine were also identified by CE-TOF-MS (*14*).

GM and Non-GM Sample Discrimination. The ^{1}H spectra of GM and non-GM seed extracts, see **Figure 1A**,**B**, are conservative because all of the signals are maintained in both genotypes, suggesting that transgenic organisms did not produce novel metabolites. Conservative ^{1}H spectra were also

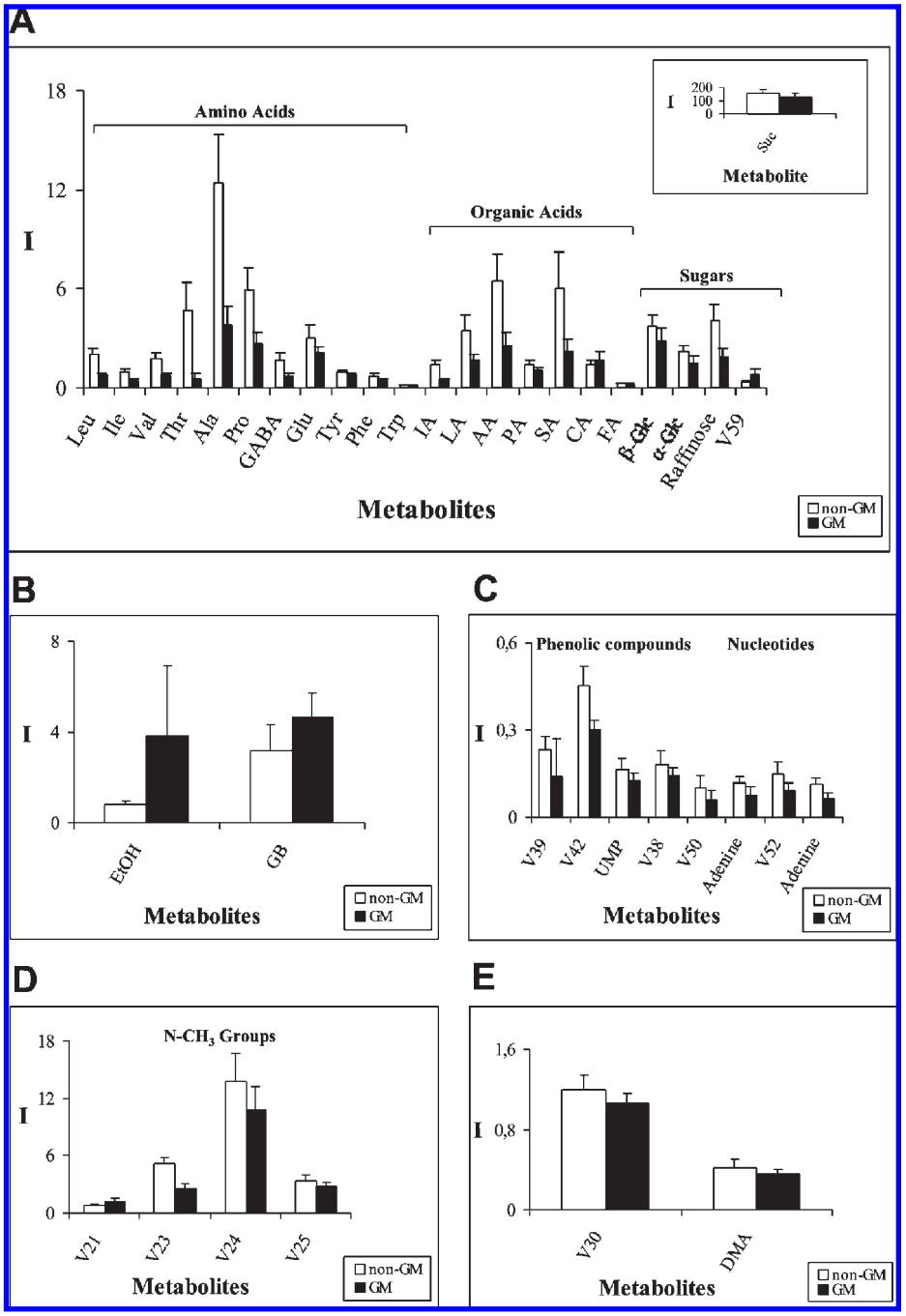

Figure 3. (**A**−**E**) Histograms relative to the intensity of selected metabolites (arbitrary units); see list of abbreviations in **Table 1**. Mean values and standard errors for GM and non-GM maize seed samples are reported. White and black bars refer to non-GM and GM maize seed samples, respectively.

observed in the case of transgenic tomato and transgenic lettuce extracts (*8*, *9*) as well as in *Zea mays* seed (*10*). In the grains of "*Zea mays*", it has previously been shown using metabolomic techniques (*10*, *14*) that genetic modification, obtained through *Cry1A(b)* gene insertion, induces unexpected metabolomic variations in pathways affecting osmolyte and branched amino acid concentrations.

In our study, to investigate whether it is possible to differentiate between GM and non-GM on the basis of differing metabolite concentrations, the NMR data were analyzed using the mixed *metabolic profiling−metabolomics* approach previously reported (*22*). In fact, although a complete assignment of the ^{1}H spectra was performed, we prefer not to define *metabolomics* as our analytical approach because some metabolites present in extremely low concentrations could not be revealed due to the intrinsic sensitivity limit of the NMR technique. The detection limit of a given metabolite, analyzed in a 5 mm tube using one-dimensional ^{1}H NMR spectroscopy at high field (11−16 T), is in the ∼10−100 μM (*4*) range. Therefore, the intensity of 53 ^{1}H signals was measured in GM and non-GM samples and submitted to ANOVA to select variables with the highest discriminative power; 39 variables were selected according to their *F* values and *p*-levels, see **Table 2**.

The mean values and standard errors of the intensity of these 39 variables were reported as histograms in **Figure 3A−E**. It is worth noting that the concentration of amino acids is always lower in the transgenic samples than in non-GM samples. The organic acids, i.e., isobutyric, lactic, acetic, pyruvic, succinic, and fumaric acids, are present in lower amounts in the transgenic samples compared with the nontransgenic ones. Only citric acid shows a higher concentration in GM samples than in the non-GM samples. All the sugars, except trehalose, are present at a lower concentration in the transgenic samples than in non-GM samples. It is important to underline that different results have been reported for seeds of variety La73-*Bt* (*10*), where the concentrations of GABA, glutamine, succinic acid, and some sugars such as α glucose, β glucose, melibiose, and sucrose have been found to be higher in GM samples than in the non-GM maize samples. This result confirms that different lines can give rise to a different metabolic profile.

The intensity of the 39 variables selected by ANOVA was submitted to PCA, see **Figure 4A**. This map clearly shows two distinct groups of samples, namely on the left side, one group consisting of non-GM samples and, on the right side, a group of only GM samples.

PC1 and PC2 account for 72.3% of the total variance, with PC1 responsible for 63.9%. The plot of variable loadings, reported in **Figure 4B**, gives important information about the discriminative power of the variables. Variable 58 (glycine-betaine), variable 59 (trehalose), variable 16 (citric acid), and

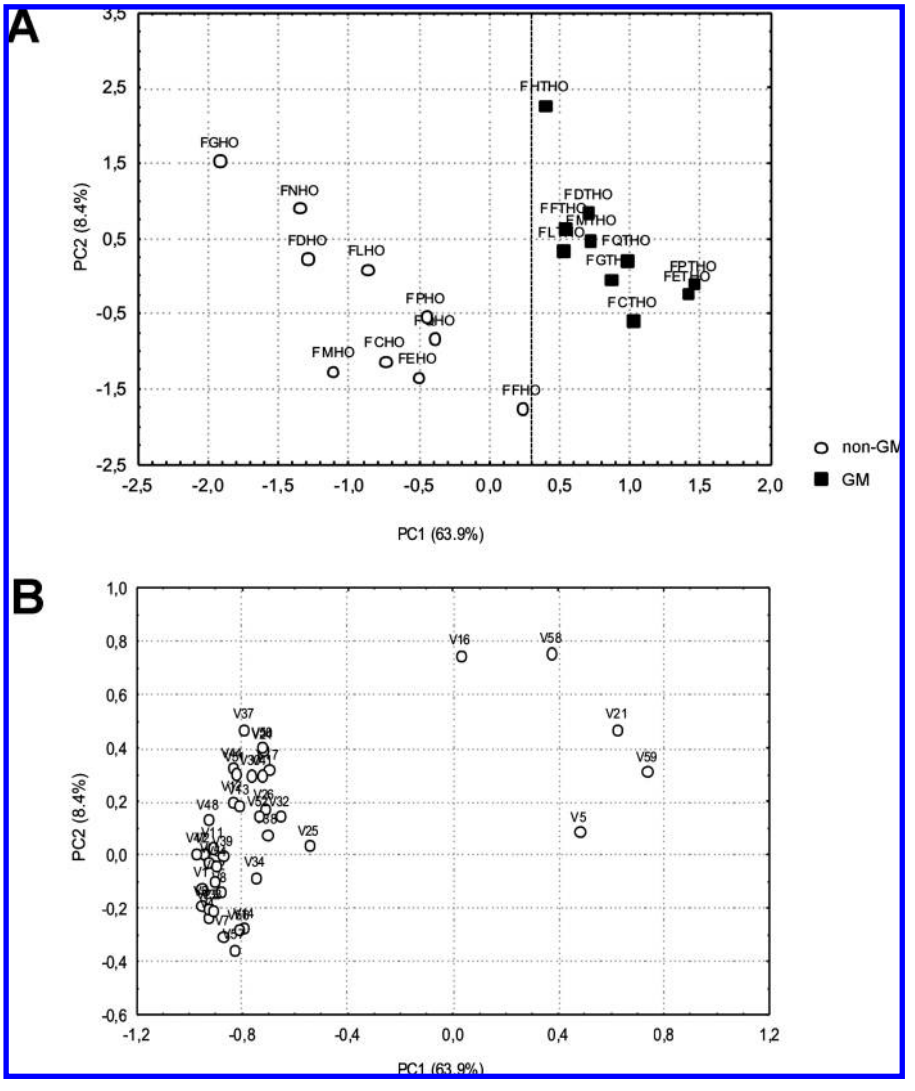

Figure 4. (**A**) PCA performed on the intensity of 39 metabolites measured in GM and non-GM samples. (**B**) PCA plot of loadings.

variable 5 (ethanol) are compounds present in higher amounts in transgenic samples as well as variable 21, which has not yet been completely assigned. At the same time, the other variables correspond to compounds present in a greater amount in non-GM extracts.

It is important to discuss in more detail those variables that increased in transgenic maize 33P67 extracts. GB (glycine-betaine) is synthesized either by oxidation (or dehydrogenation) of choline or by the *N*-methylation of glycine. It accumulates in the chloroplasts and plastids of many halotolerant plants, resulting in resistance to this abiotic stress. Studying salt tolerance of glycinebetaine-deficient and -containing maize lines, it has been also reported (*33*) that a single gene, conferring glycine-betaine accumulation (and/or a tightly linked locus), plays a key role in osmotic adjustment in maize leaves.

Trehalose is another osmolyte present in a wide variety of organisms (including yeast, fungi, insects, lower and higher plants) (*34*) and functions protecting plants from stress and storing carbohydrates (*35*). The biosynthesis of trehalose has been best studied in *Escherichia coli* and *Saccharomyces cerevisiae* and involves a two-step process catalyzed by trehalose-6-phosphate synthase and trehalose-6-phosphate-phosphatase. Trehalose-6-phosphate is formed from glucose-6-phosphate and uridine-5-diphosphoglucose by trehalose-6-phosphate synthase and is then dephosphorylated to trehalose by trehalose-6-phosphate-phosphatase (*36*, *37*). It is an essential component of the mechanisms that coordinate metabolism during plant growth adaptation and development. Trehalose is known to accumulate in high concentrations to cope with complete dehydration in anhydrobiotic organisms (*38*), preserving membranes during drought periods (*39*). This property makes trehalose an interesting candidate for genetic engineering in order to create highly stress-resistant plants (*40*). Ethanol increase in GM samples can be attributed to an alteration of the glycolytic pathway (transformation of pyruvate into ethanol) and of the Krebs's cycle. The alteration of the Krebs's cycle in the transgenic samples is also suggested by the decrement of succinic and fumaric acid content and by the increase of citric acid. The same trend was observed in the case of the organic acids present in transgenic and nontransgenic lettuce (*9*).

The high production of osmolytes "glycine-betaine and threalose" revealed in this line was not detected previously in another maize line (*10*). Osmolytes such as L-carnitine and L-prolinebetaine (stachydrine) have been identified in the maize line 33P67 (*14*) using the CE-TOF-MS approach in order to screen transgenic maize metabolome.

The alterations in the concentration of some metabolites, revealed in the present study, could take origin from several factors. Among these factors, genetic mutations could be involved as a consequence of particle bombardment technique. This is a random process, which can lead to the physical disruption of the genome or alteration in gene regulation, with a potential inactivation of endogenous genes (*15*). Upon transformation event, somaclonal mutations could take place; this term indicates the generation of spontaneous and heritable genetic changes in in vitro cultures (*41*). This is particularly true if one or more heritable mutations were closely linked to the transgene or to genes controlling other critical traits, a situation which would limit the value of the genetic engineering approach. The net involvement of this factor in the alteration of metabolic profile, which has not been elucidated in the present paper, will be an interesting starting point for further deepenings.

Other factors potentially responsible for the metabolic alteration could be pleiotropic effects: in fact, the introduction of an external sequence can influence not only the proximal region of insertion, where modifications are due to reshuffling of genetic sequences to include the new genetic fragment, but also in distal regions of the genome, affecting the whole equilibrium of pathways (*15*). Pleiotropic effects cannot be excluded, although no novel compounds appeared in the transgenic line .

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation; HSQC heteronuclear single quantum coherence; DOSY, diffusion ordered spectroscopy; CE-TOF-MS, capillary electrophoresis time-of-flight mass spectrometry; GM, genetically modified; ANOVA, analysis of variance; PCA principal component analysis.

LITERATURE CITED

(1) Cellini, F.; Chesson, A.; Colquhoun, I.; Constable, A.; Davies, H. V.; Engel, K. H.; Gatehouse, A. M. R.; Karenlampi, S.; Kok, E. J.; Leguay, J. J.; Lehesranta, S.; Noteborn, H. P.; Pedersen, J.; Smith, M. Unintended effects and their detection in genetically modified crops. *Food Chem. Toxicol.* **2004**, *42*, 1089–1125.

(2) Chassy, B.; Hlywka, J. J.; Kleter, G. A.; Kok, E. J.; Kuiper, H. A.; McGloughlin, M.; Munro, I. C.; Phipps, R. H.; Reid, J. E. Nutritional and safety assessments of foods and feeds nutritionally improved through biotechnology: an executive summary. *Compr. Rev. Food Sci. Food Saf.* **2004**, *3*, 38–104.

(3) Le Gall, G.; Colquhoun, I. J.; Davis, A. L.; Collins, G. J.; Verhoeyen, M. E. Metabolite profiling of tomato (*Lycopersicon esculentum*) using ^{1}H NMR spectroscopy as a tool to detect potential unintended effects following a genetic modification. *J. Agric. Food Chem.* **2003**, *51*, 2447–2456.

(4) Fan, T.M.-W. Metabolite profiling by one-and two-dimensional NMR analysis of complex mixtures. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *28*, 161–219.

(5) Dabos, K. J.; Nelson, L. J.; Bradnock, T. J.; Parkinson, J. A.; Sadler, I. H.; Hayes, P. C.; Plevris, J. N. The simulated microgravity environment maintains key metabolic functions and promotes aggregation of primary porcine hepatocytes. *Biochim. Biophys. Acta, Gen. Subj.* **2001**, *1256*, 119–130.

(6) Krishnan, P.; Kruger, N. J.; Ratcliffe, R. G. Metabolite fingerprinting and profiling in plants using NMR. *J. Exp. Bot.* **2005**, *56*, 255–265.

(7) Govindaraju, V.; Young, K.; Maudsley, A. A. Proton NMR chemical shifts and coupling constants for brain metabolites. *NMR Biomed.* **2000**, *13*, 129–153.

(8) Mattoo, A. K.; Sobolev, A. P.; Neelam, A.; Goyal, R. K.; Handa, A. K.; Segre, A. L. Nuclear magnetic resonance spectroscopy-based metabolite profiling of transgenic tomato fruit engineered to accumulate spermidine and spermine reveals enhanced anabolic and nitrogen−carbon interactions. *Plant Physiol.* **2006**, *142*, 1759–1770.

(9) Sobolev, A. P.; Segre, A. L.; Giannino, D.; Mariotti, D.; Nicolodi, C.; Brosio, E.; Amato, M. E. Strong increase of foliar inulin occurs in transgenic lettuce plants (*Lactuca sativa* L.) overexpressing the *Asparagine synthetase A* gene from *Escherichia coli*. *J. Agric. Food Chem.* **2007**, *55*, 10827–10831.

(10) Manetti, C.; Bianchetti, C.; Casciani, L.; Castro, C.; Di Cocco, M. E.; Miccheli, A.; Motto, M.; Conti, F. A metabonomic study of transgenic maize (*Zea mays*) seeds revealed variations in osmolytes and branched amino acids. *J. Exp. Bot.* **2006**, *57*, 2613–2625.

(11) Castro, C.; Manetti, C. A multiway approach to analyze metabonomic data: a study of maize seeds development. *Anal. Biochem.* **2007**, *371*, 194–200.

(12) Castro, C.; Motto, M.; Rossi, V.; Manetti, C. Variation of metabolic profiles in developing maize kernels up- and down-regulated for the *hda101* gene. *J. Exp. Bot.* **2008**, *59*, 3913–3924.

(13) Zolla, L.; Rinalducci, S.; Antonioli, P.; Righetti, P. G. Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. *J. Proteome Res.* **2008**, *7*, 1850–1861.

(14) Levandi, T.; Leon, C.; Kaljurand, M.; Garcia-Canas, V.; Cifuentes, A. Capillary electrophoresis time-of-flight mass spectrometry for comparative metabolomics of transgenic versus conventional maize. *Anal. Chem.* **2008**, *80*, 6329–6335.
(15) Somers, D. A.; Makarevitch, I. Transgene integration in plants: poking or patching holes in promiscuous genomes. *Curr. Opin. Biotechnol.* **2004**, *15*, 126–131.
(16) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem.* **1959**, *37*, 911–917.
(17) Miccheli, A.; Aureli, T.; Delfini, M.; Di Cocco, M. E.; Viola, P.; Gobetto, R.; Conti, F. Study on influence of inactivation enzyme techniques and extraction procedures on cerebral phosphorylated metabolite levels by 31P NMR spectroscopy. *Cell. Mol. Biol.* **1988**, *34*, 591–603.
(18) Braun, S.; Kalinowski, H.-O.; Berger, S. *150 and More Basic NMR Experiments*, 2nd ed.; Wiley-VCH: Weinheim, Germany, 1998.
(19) Mannina, L.; Cristinzio, M.; Sobolev, A. P.; Ragni, P.; Segre, A. L. High-Field Nuclear Magnetic Resonance (NMR) Study of Truffles (*Tuber aestivum vittadini*). *J. Agric. Food Chem.* **2004**, *52*, 7988–7996.
(20) Nicholson, J. K.; Foxall, P. J.; Spraul, M.; Farrant, R. D.; Lindon, J. C. 750 MHz ^{1}H and ^{1}H−^{13}C NMR spectroscopy of human blood plasma. *Anal. Chem.* **1995**, *67*, 793–811.
(21) Piccioni, F.; Borioni, A.; Delfini, M.; Del Giudice, M. R.; Mustazza, C.; Rodomonte, A.; Risuleo, G. Metabolic alterations in cultured mouse fibroblasts induced by an inhibitor of the tyrosine kinase receptor FGFR-1. *Anal. Biochem.* **2007**, *367*, 111–121.
(22) Mannina, L.; Sobolev, A. P.; Capitani, D.; Iaffaldano, N.; Rosato, M. P.; Ragni, P.; Reale, A.; Sorrentino, E.; D'Amico, I.; Coppola, R. NMR metabolic profiling of organic and aqueous sea bass extracts: implication in the discrimination of wild and cultured sea bass. *Talanta* **2008**, *77*, 433–444.
(23) Sobolev, A. P.; Segre, A. L.; Lamanna, R. Proton high-field NMR study of tomato juice. *Magn. Reson. Chem.* **2003**, *41*, 237–245.
(24) Morris, K. F.; Johnson, C. S. Diffusion-ordered two-dimensional nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc.* **1992**, *114*, 3139–3141.
(25) Morris, K. F.; Stilbs, P.; Johnson, C. S. Analisys of mixtures based on molecular size and hydrophobility by means of diffusion ordered 2D-NMR. *Anal. Chem.* **1994**, *66*, 211–215.
(26) Kapur, G. S.; Cabrita, E. J.; Berger, S. The qualitative probing of hydrogen bond strength by diffusion-ordered NMR spectroscopy. *Tetrahedron Lett.* **2000**, *41*, 7181–7185.
(27) Viel, S.; Mannina, L.; Segre, A. L. Detection of a $\pi-\pi$ complex by diffusion-ordered spectroscopy (DOSY). *Tetrahedron Lett.* **2002**, *43*, 2515–2519.
(28) Bachmann, M.; Matile, P.; Keller, F. Metabolism of the raffinose family oligasaccharides in leaves of *Ajuga reptans L.* (cold acclimation, translocation, and sink to source transition: discovery of chain elongation enzyme). *Plant Physiol.* **1994**, *105*, 1335–1345.
(29) Haritatos, E.; Keller, F.; Turgeon, R. Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L. leaves: implications for phloem loading. *Planta* **1996**, *198*, 614–622.
(30) Taji, T.; Ohsumi, C.; Iuchi, S.; Seki, M.; Kasuga, M.; Kobayashi, M.; Yamaguchi-Shinozaki, K. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tollerance in *Arabidopsis thaliana*. *Plant J.* **2002**, *29*, 417–426.
(31) Blochl, A.; Peterbauer, T.; Richter, A. Inhibition of raffinose oligisaccharide breakdown delays germination of pea seeds. *Plant Physiol.* **2007**, *164*, 1093–1096.
(32) Tolonen, A.; Joutsamo, T.; Mattla, S.; Kamarainen, T.; Jalonen, J. Identification of isomeric dicaffeoylquinic acids from *Eleutherococcus senticosus* using HPLC-ESI/TOF/MS and ^{1}H NMR methods. *Phytochem. Anal.* **2002**, *13*, 316–328.
(33) Saneoka, H.; Nagasaka, C.; Hahn, D. T.; Yang, W.; Premachandra, G. S.; Joli, R. J.; Rhodes, D. Salt tolerance of glycinebetaine-deficient and -containing maize lines. *Plant Physiol.* **1995**, *107*, 631–638.
(34) Elbein, A. D. The metabolism of a,a-trehalose. *Adv. Carbohydr. Chem. Biochem.* **1974**, *30*, 227–256.
(35) Goddijin, O. J. M.; Van Duc, K. Trehalose metabolism in plants. *Trends Plant Sci.* **1999**, *4*, 315–319.
(36) Eastmond, P. J.; Li, Y.; Graham, I. A. Is trehalose-6-phosphate a regulator of sugar metabolism in plants?. *J. Exp. Bot.* **2003**, *54*, 533–537.
(37) Cabib, E.; Leloir, L. F. The biosynthesis of trehalose phosphate. *J. Biol. Chem.* **1958**, *231*, 259–275.
(38) Drennan, P. M.; Smith, M. T.; Goldsworthy, D.; Van Staden The occurrence of trehalose in the leaves of the desiccation-tolerant angiosperm *Myrothamnus flabellifolius* Welw. *Plant Physiol.* **1993**, *142*, 493–496.
(39) Crowe, J. H.; Crowe, L. M.; Chapman, D. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **1984**, *223*, 701–703.
(40) Goddijn, O. J. M.; Verwoerd, T. C.; Voogd, E.; Krutwagen, R. W. H. H.; de Graaf, P. T. H. M.; Poels, J.; van Dun, K.; Ponstein, A. S.; Damm, B.; Pen, J. Inhibition of trehalose activity enhances trehalose accumulation in transgenic plants. *Plant Physiol.* **1997**, *113*, 181–190.
(41) Larkin, P. J.; Scowcroft, W. R. Somaclonal variation: A novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* **1981**, *60*, 197–214.

Received March 10, 2009. Revised manuscript received May 27, 2009. Accepted May 30, 2009.