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Strong Increase of Foliar Inulin Occurs in Transgenic Lettuce Plants (*Lactuca sativa* L.) Overexpressing the *Asparagine Synthetase A* Gene from *Escherichia coli*

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Transgenic lettuce (*Lactuca sativa* L. cv. 'Cortina') lines expressing the *asparagine synthetase A* gene from *Escherichia coli* were produced to alter the plant nitrogen status and eventually enhance growth. The relative molecular abundance of water-soluble metabolites was measured by ^{1}H NMR in transgenic and conventional plants at early developmental stages and grown under the same conditions. NMR metabolic profiles assessed that a transgenic line and the wild-type counterpart shared the same compounds, but it also revealed side effects on the carbon metabolism following genetic modification. Concerning the nitrogen status, the amino acid content did not vary significantly, except for glutamic acid and γ-aminobutyric acid, which diminished in the transgenics. As for the carbon metabolism, in transgenic leaves the contents of sucrose, glucose, and fructose decreased, whereas that of inulin increased up to 30 times, accompanied by the alteration of most Krebs's cycle organic acids and the rise of tartaric acid compared to nontransformed controls. Lettuce leaf inulins consisted of short oligomeric chains made of one glucose unit bound to two/four fructose units. Inulins are beneficial for human health, and they are extracted from plants and commercialized as long-chain types, whereas the short forms are synthesized chemically. Hence, lettuce genotypes with high content of foliar short-chain inulin represent useful materials for breeding strategies and a potential source for low molecular weight inulin.

KEYWORDS: Lettuce; transgenic; NMR; metabolite profiling; foliar inulin; short-chain oligofructans

INTRODUCTION

The "substantial equivalence" is the internationally recognized standard that measures whether a biotech food shares similar health and nutritional characteristics with its conventional counterpart (*1, 2*). High-resolution NMR proved to be a powerful technique to identify and quantify a large number of compounds in foods and to accurately assess the differences, including undesired effects, between genetically modified plants (GMPs) and conventional varieties (*3*). An NMR spectrum is able to fully picture the complex mixtures of metabolites and does not require time-consuming separation and/or derivatization of components (*4–7*). The proper statistical analysis of NMR spectra is a reliable support to evaluate the quality and safety of GMP-based foodstuff (*8*), responding to specific public demands (*9*).

Lettuce plants expressing the *asaparagin synthese A* (*asnA*) gene are characterized by a higher leaf mass and dry weight and quicker bolting and flowering than nontransgenic controls (*10*). They also showed the increase of a few amino acids involved in long-distance N transport and overall protein and chlorophyll *a* contents. It has been proposed that bacterial *asnA* ectopic expression may enhance N metabolism and favor CO_2 assimilation, considering that the C and N metabolic pathways are reciprocally regulated by several overlapping branch points during organ development (*11*). NMR metabolic profiling of the water-soluble fraction was performed to compare leaves of

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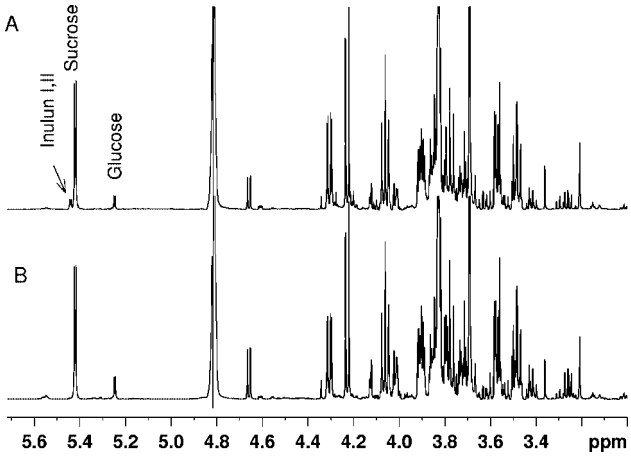

Figure 1. ^{1}H NMR spectra of water-soluble fraction of wild lettuce leaves at (**A**) 3–4 leaf and (**B**) 6–8 leaf developmental stages. Only the spectral region spanning from 3 to 5.6 ppm is shown.

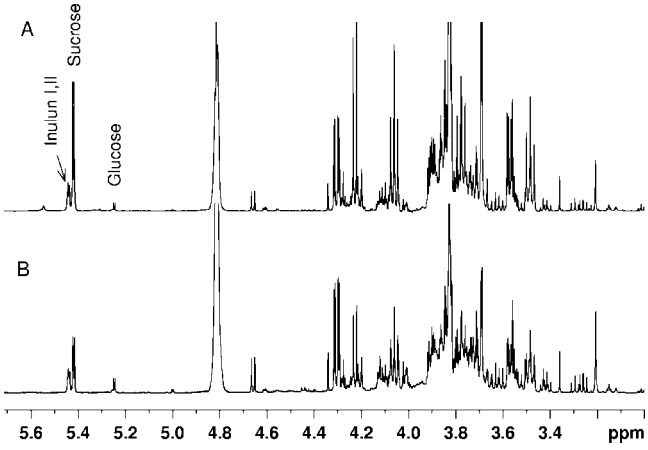

Figure 2. ^{1}H NMR spectra of water-soluble fraction of transgenic leaves at (**A**) 3–4 leaf and (**B**) 6–8 leaf developmental stages. Only the spectral region spanning from 3 to 5.6 ppm is shown.

transgenic lettuce expressing the bacterial *asparagine synthetase A* (*10*) with the nontransformed controls.

Interestingly, a dramatic increase of the inulin (1-kestose, G-F_2 type, and inulin G-F_4 type) content was observed in transgenic leaves. This appeared as an unexpected effect because the transgene aims to modify the asparagine content, together with the N status, rather than that of carbohydrates.

Inulins are plant storage carbohydrates mainly found in Asteraceae species (*12*), representing natural food ingredients (*13*). The nutritional properties range from the replacement of fat and sugar in dietary fibers to the stimulation of *Bifidobacteria* activity etc. (*14*), responding to consumers' demand for low caloric and tasty food with health benefits. The high solubility and sweet taste of short-chain inulins (oligofructoses) compared to the neutral clean flavor and low solubility of long-chain inulins account for their distinctly different use in the food industry. From a chemical point of view, 1-kestose (G-F_2 type) is the shortest oligomer representative of inulins (*15*). In the industrial food market oligofructoses are either synthesized from sucrose (*16*) or extracted from chicory roots and Jerusalem artichoke followed by partial enzymatic hydrolysis of long-chain inulins (*17*).

MATERIALS AND METHODS

Plant Growth Condition and Sampling. Wild-type and transgenic lines of *Lactuca sativa* cv. 'Cortina' (long-day butter head lettuce, provided by Nunhems, www.nunhems.com) overexpressing *Escherichia coli asnA*, under the hybrid constitutive *pMAC* promoter, were produced (*pMAC:asnA* genotype) and cultivated as previously described (*10*). In this study we used the progeny (T_6 generation) derived from T_0 B14, containing a putative single T-DNA locus (*10*). All of the T_6 B14 individuals showed the large-leaf phenotype (T-DNA homozygosis was reached), and 10 samples were confirmed to express the *asnA* transcript at the A and B developmental stages (four and six total leaves, respectively). Seeds of both transgenic and conventional 'Cortina' were sown at the beginning of April 2004, and sampling occurred in the morning (9:00 a.m.) after 3 and 4 weeks for A and B stages, respectively. Because the plastochrone (leaf number per day) of *pMAC:asnA* genotypes was higher than that of the wild type (*10*), the leaves of the latter were sampled ca. 48 h later than the transgenics, to ensure that the leaf numbers were equal between transgenic and wild-type samples. Five hundred milligrams of fresh leaves produced ca. 30 mg of lyophilized material (6%), regardless of the developmental stage. At stage A, whole aerial parts of seedlings were collected; at stage B, a pool of fully expanded leaves ($n = 3$) was made for each distinct plant. Fifteen plants were assayed at stage A and 20 at stage B, making a total of 35 samples.

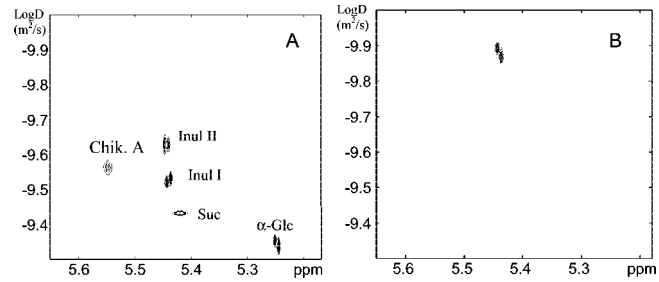

Figure 3. DOSY spectra: (**A**) aqueous extract from transgenic lettuce leaves; (**B**) commercial inulin. α-Glc, D-α-glucose; Suc, sucrose; Inul I, inulin I; Inul II, inulin II; Chik, chicoric acid. *Y* and *X* axes report the logarithm of diffusion coefficient and the ^{1}H chemical shift, respectively.

NMR Spectroscopy. NMR metabolic profiles (including 1-kestose and inulin) in lettuce were carried out according to a procedure reported previously (*7*). The water-soluble extract was prepared according to the following procedure: 1 mL of a D_2O phosphate buffer, having 400 mM salt concentration and pD value of 6.5 and containing 0.1 mM 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSPA) and 0.05 mM EDTA, was mixed with 21.0 mg of powdered vegetal tissues. The mixture was centrifuged at 10000 rpm for 7 min; supernatant was filtered and transferred in a standard 5 mm NMR tube.

NMR spectra of lettuce extracts were recorded at 300 K on a Bruker AVANCE AQS600 spectrometer operating at the ^{1}H frequency of 600.13 MHz and equipped with a Bruker multinuclear *z*-gradient inverse probehead capable of producing gradients in the *z* direction with a strength of 55 G cm^{-1}. Proton spectra were referenced to methyl groups signal at $\delta = 0.00$ of TSPA in D_2O. ^{1}H spectra of aqueous extracts were acquired by co-adding 512 transients with a recycle delay of 3 s and 32K data points. The residual HDO signal was suppressed using a presaturation with a long single soft pulse. To avoid possible saturation effects on unknown compounds, the experiment was carried out by using a 45° flip angle pulse of 8.0 μs.

DOSY spectra were obtained using a bipolar LED sequence with sine-shaped gradient (*18*) elaborated by methods as previously described (*19*). The following experimental settings were used: diffusion time, Δ, was 200 ms; gradient duration, $\delta/2$, was 1.6 ms, and the longitudinal eddy current delay was 25 ms.

The abundances of inulins I and II were determined by the integration and deconvolution of the multiplet at 5.44 ppm arising from the overlapping of two doublets due to anomeric proton of glucose units.

Statistical Analyses. The data sets concerning the inulin contents were subjected to Student's *t* test, and those presented in this work have a $p < 0.05$, except where specified. Signal intensities in ^{1}H NMR spectra corresponding to selected metabolites (determined as peak

Table 1. ANOVA Results for Selected Signals from *pMAC:asnA* and Wild-Type Lettuce Spectra

compound[a]	*p* level	*F* value	degree of alteration[b]	order[c]
valine (1.05)[d]	0.48	0.51		
isoleucine (1.02)	0.72	0.14		
threonine (1.32)	0.11	2.71		
alanine (1.49)	0.86	0.03		
glutamic acid (2.07)	0.013	6.88	1.4	c > t
glutamine (2.45)	0.44	0.61		
aspartic acid (2.81)	0.54	0.38		
asparagine (2.89)	0.14	2.31		
γ-aminobutyric acid (2.30)	0.0026	10.56	1.7	c > t
malic acid (2.39)	0.0061	8.59	1.2	c > t
succinic acid (2.41)	0.0026	10.60	1.4	c > t
citric acid (2.54)	0.00014	18.44	1.7	c < t
α-ketoglutaric acid (3.01)	0.00027	16.61	1.4	c > t
tartaric acid (4.34)	0.0022	11.05	1.6	c < t
fumaric acid (6.51)	0.000031	23.23	2.1	c > t
acetic acid (1.93)	0.076	3.34		
glucose (3.26)	0.00018	17.86	1.8	c > t
fructose (4.02)	0.000008	28.14	2.2	c > t
sucrose (5.42)	0.0000001	47.37	1.9	c > t
inulin I (5.439)	0.000005	29.28	3.3	c << t
inulin II (5.445)	0.0000001	52.52	6.9	c << t
choline (3.21)	0.0022	10.99	1.25	c > t
myo-inositol (3.29)	0.50	0.46		
methanol (3.36)	0.85	0.03		

[a] Extracts form the water-soluble fraction; the significant variables with $p < 0.02$ are bolded; the ^{1}H chemical shifts of the corresponding resonances (in ppm) are given in parentheses. [b] Reported in fold times only when significant at the statistical level. [c] c, control; t, transgenic. [d] The ^{1}H chemical shifts of the corresponding resonance (in ppm) is given in parentheses.

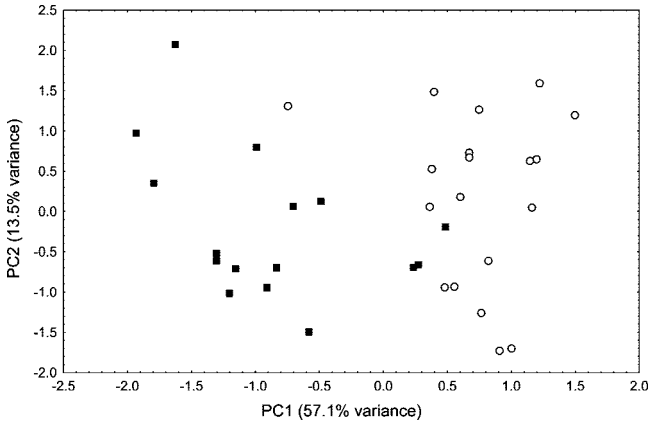

Figure 4. PCA scatterplot of wild (■) and GM (○) lettuce samples. PCA was performed using the following variables: glutamic acid, γ-aminobutyric acid, malic acid, succinic acid, citric acid, α-ketoglutaric acid, tartaric acid, fumaric acid, glucose, fructose, sucrose, inulin I, inulin II, and choline.

heights of individual signals) were normalized in reference to the standard (TSPA) and subjected to statistical analysis by using Statistica software package for Windows (1997; edition by Statsoft).

ANOVA (*20*) proves that the null hypothesis (i.e., no statistically significant differences between the variances of the groups) for the selected resonances is not valid. The results of this analysis are reported as *F* and *p* level values. The *F* value, with the degrees of freedom test, measures whether the between and within variances are significantly different. The *p* level represents a decreasing index of the reliability of a result and gives the probability of error involved in accepting a result as a reliable one. Principal component analysis (PCA) results in a linear combination of the original variables to yield a few principal components with the highest variance and so drastically reduces the number of variables to be analyzed (*20*). Unless specified, PC scores are used instead of the original variables.

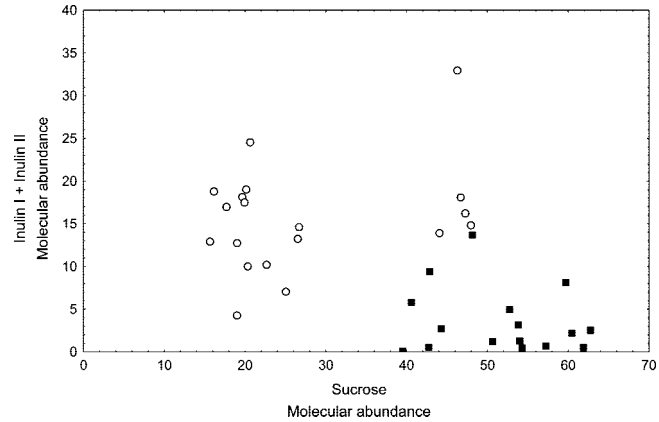

Figure 5. Sum of inulin I and inulin II molecular abundances versus that of sucrose: wild (■) and GM (○) lettuce samples.

RESULTS AND DISCUSSION

The ^{1}H NMR spectra of wild-type (**Figure 1**) and *pMAC:asnA* (**Figure 2**) leaf pools were carried out using lettuce at the A and B (early) developmental stages. Spectra were conservative because all of the specific signals were maintained in both genotypes, although the intensity varied with the stages. At a given developmental stage, a set of peaks was observed to constantly differ between the two genotypes. Moreover, NMR did not reveal any new signal, suggesting that the transgenics did not produce novel metabolites and that they can be "equivalent" to those of the conventional variety. All resonance peaks were previously assigned for specific compounds of lettuce (*7*).

Inulin I and II signals partially overlapped; hence, they were further separated by the pulse field gradient method (*18*) and represented in a two-dimensional diagram (**Figure 3**), plotting the ^{1}H spectrum against the diffusion coefficient (*D*). The molecular weights (M_w) of inulins I and II were calculated by comparing their diffusion coefficients with that of sucrose, assuming that *D* is proportional to $M_w^{-0.49}$ according to the empirical equation $D = 8.2 \times 10^{-9} \times M_w^{-0.49}$ (in $m^2\ s^{-1}$) established for neutral polysaccharides (*19*). The prevailing forms synthesized in leaf lettuce at A and B stages were 1-kestose G-F_2 (inulin I) and G-F_4 oligomer (inulin II).

The 24 major water-soluble lettuce metabolites and nutrients assigned in ^{1}H NMR spectra (*7*) were chosen for the statistical analysis. The corresponding ^{1}H NMR signals (**Table 1**) did not overlap, and the complete extraction of these metabolites from lettuce leaves was assured. The phenols were not quantified because their concentration in aqueous extracts was strongly influenced by the partial oxidation in the presence of oxygen.

ANOVA was carried out to assess which of the 24 variables could be used to discriminate significantly the GM from the wild-type individuals (**Table 1**) regardless of developmental stages. Metabolites with a *p* level of <0.02 and an *F* factor of >6 were considered to be significant and used to perform PCA (**Figure 4**), which displayed a clear separation of the wild type from the transgenic genotypes. Taking into account the low *p* level values, we further plotted the sum of inulin I and II contents versus that of sucrose (**Figure 5**). All of the wild-type samples appeared to be grouped in the area of high sucrose and low inulin contents, whereas all of the GM samples fell in the high inulin and/or low sucrose content area. Moreover, a plot of inulin II against inulin I further pictured two distinct areas for GM and wild-type plants (data not shown). Therefore, the inulin content (**Figure 5**) evidenced a significant difference between the wild-type and GM individuals, which was inde-

Table 2. Average Relative Molar Concentration (RMC) and Abundance (Q) of Inulins in GM and Wild-Type Lettuce Plants

	wild type				GM			
	inulin I		inulin II		inulin I		inulin II	
developmental stage	RMC[a]	Q[b]	RMC	Q	RMC	Q	RMC	Q
A (3–4 leaves)	3.6 ± 0.9[c]	1.20 ± 0.03	1.4 ± 0.5	0.7 ± 0.2	9.1 ± 1.0	2.7 ± 0.3	5.5 ± 0.3	2.7 ± 0.2
B (6–8 leaves)	1.0 ± 0.4	0.3 ± 0.1	0.25 ± 0.20	0.12 ± 0.01	8.4 ± 1.1	2.5 ± 0.3	7.5 ± 0.9	3.7 ± 0.4

[a] RMC was directly determined by NMR using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSPA) as internal reference. [b] Milligrams per gram of fresh weight. [c] Standard error.

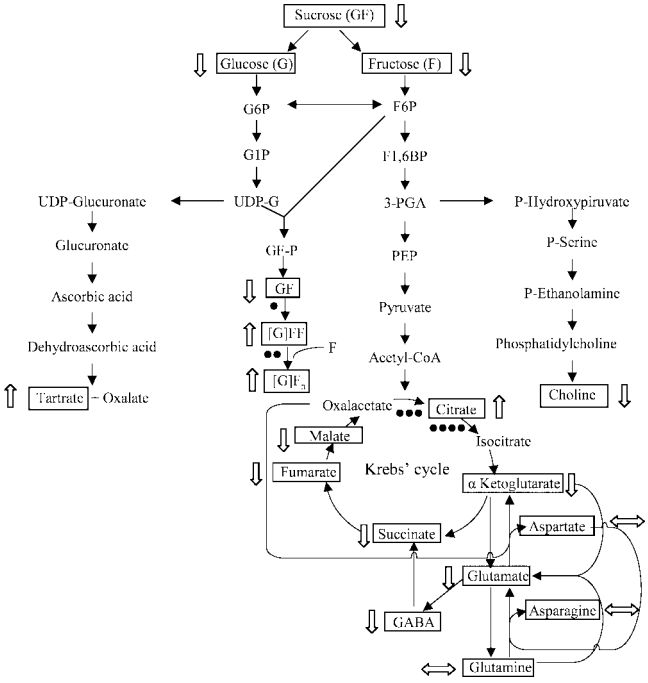

Figure 6. Scheme of biosynthetic pathways of metabolites identified by NMR (boxed). Increased (white arrows pointing upward), decreased (white arrows pointing downward), and unchanged (horizontal arrows) metabolite contents are represented for transgenic leaves. The alteration (activity, concentration, etc.) of a few key enzymes (positioned by black circles in the pathways) might account for metabolite content changes in GM plants. •, sucrose:sucrose 1-fructosyltransferase; ••, fructan:fructan 1-fructosyltransferase; •••, citrate synthase; ••••, aconitase; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; UDP-G, uridine diphosphoglucose; UDP-Glucuronate, uridine diphosphoglucuronate; GF-P, sucrose phosphate; [G]FF, 1-kestose; [G]F_n, inulins; F6P, fructose-6-phosphate; F1,6BP, fructose 1,6-biphosphate; 3-PGA, 3-phosphoglyceric acid; PEP, phosphoenol pyruvate; GABA, γ-aminobutyric acid.

pendent from the plant growth stage and likely to be caused by the transgene effect.

The average relative molar concentration (RMC) and quantity of inulins I and II varied with developmental stages in both genotypes (**Table 2**), but they were consistently higher in GM than in conventional leaves. More precisely, inulin I concentration was from 2.5- to 8.5-fold higher in GM than in control leaves, whereas inulin II concentration was from 4- to 30-fold higher. The content of short inulins ($GF_{3–10}$) has been mostly determined in Asteraceae roots: it ranged around 60 mg g^{-1} of fresh weight in chicory roots and varied according to several parameters such as cultivar, sowing and harvesting dates, etc. (*21*). We were not able to retrieve literature data on the inulin content in lettuce leaves; however, total fructans content of large leaves from chicory at harvesting was measured to vary from 3 to 6 mg g^{-1} of dry weight (*22*). Young leaves of *pMAC:asnA* lettuce plants at stage B contained ca. 6 mg g^{-1} of fresh weight (**Table 2**), which turns out to be a remarkable content of ca. 100 mg g^{-1} of dry weight (average water content of lettuce leaf was ca. 94%, see Materials and Methods).

In transgenic genotypes, the inulins rise was concomitant with a significant decrease of fructose, sucrose, and glucose (**Figure 6**). The high levels of inulins was accompanied by a low level of di- and monosaccharides, and this may imply the enhanced efficiency of sucrose 1-fructosyl and fructan 1-fructosyltransferases (**Figure 6**), which are crucial inulin-polymerizing enzymes (*23*). The variation of concentration and/or kinetics of these enzymes was not addressed in this work. However, the occurrence of only short-chain inulins strongly supports the hypothesis that a synthesis rather than a catabolic process was enhanced in the transgenics. In addition, the content of tartaric acid, which derives from sucrose remetabolization (*24*), was higher in GM than in wild-type plants. Overall, the augmentations of inulin and tartaric acid levels were consistent with the intensive catabolism of mono- and disaccharides. The ANOVA also showed that Krebs's cycle was altered: the content of α-ketoglutaric, succinic, fumaric, and malic acids decreased, whereas that of citric acid increased (**Figure 6**). The latter event leads to the hypothesis that the regulation of citrate synthase and/or aconitase activities might have been affected, which, again, was not addressed in this work.

Concerning the amino acids, significant differences occurred for glutamic and γ-aminobutyric acid contents when the two genotypes were compared, suggesting that there was a transgene effect in altering the amino acid metabolism and the N status, consistent with previously reported results (*10*). In this study, however, the contents of N transporter amino acids (Asn, Asp, and Gln) appeared not to vary significantly in T_6 B14 *pMAC:asnA* genotypes, in contrast with the amino acid alterations (revealed by HPLC methods) observed in T_2 B14 *pMAC:asnA* individuals, which included the ancestor of T_6 B14 (*10*). Given that nutritional conditions were the same in previous and present experiments and that T_6 B14 individuals produced the *asnA* mRNA, a reason for the discrepancy may be attributed to the different sampling timing, which occurred 3 weeks after sowing in late May in previous studies (*10*), whereas NMR analyses were performed on plants sown in early April. The cultivar 'Cortina' is a long-day variety, and lettuce nitrogen metabolism, including that of amino acids, is known to be regulated by light (*25*) as in most plants (*26*). Plant metabolite contents are known to vary greatly according to environmental factors (*27*); hence, these may modulate the transgene downstream effects (rather than its transcription) acting on the amino acid composition in genetically modified 'Cortina'.

In this study, NMR-based fingerprinting allowed the monitoring of a wide range of metabolic alterations, including those unexpected/unpredicted, in transgenic lettuce plants. Nonetheless, our results showed that the *pMAC:asnA* lettuce line is of interest for breeding programs to improve varieties producing inulins. Other prospects for this transgenic line may include either the use of inulin-enriched foliage for dietary food (e.g.,

packaged salads) or the extraction of natural rather than chemically derived short inulins of plant origin (favored by the confectionery industries). Finally, NMR metabolic analyses of distinct *pMAC:asnA* 'Cortina' lines are in progress on a large size population at head harvesting. These analyses will be accompanied by mRNA profiling of target genes (as identified in **Figure 6**) and monitoring of respective enzyme activities.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; DOSY, diffusion ordered spectroscopy; G-F_n, α-D-glucopyranosyl-[β-D-fructofuranosyl] $(n-1)$-D-fructofuranoside; GM, genetically modified; ANOVA, analysis of variance; PCA, principal component analysis.

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