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ARTICLE

Quality Traits of Conventional and Transgenic Lettuce (*Lactuca sativa* L.) at Harvesting by NMR Metabolic Profiling†

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Metabolism of genetically modified (GM) lettuce (*Lactuca sativa* L.) leaves was investigated by comparing NMR metabolic profiles of three lines (T_3B12, T_7B7, and T_7B14) overexpressing the *E. coli asparagine synthetase A* gene with those of the wild type (WT) at 24, 56, and 64 days after sowing (DAS). Statistical analyses based on hydro-soluble compound profiles significantly and maximally discriminated the WT from GM-lines at optimal harvest time (56 DAS). The T_7B14 metabolic variations were opposite to those of both T_3B12/T_7B7 lines, suggesting that unexpected effects of transgenesis had occurred. Compared to controls, the T_3B12/T_7B7 plants shared the leaf mass increase, higher amino acid (asparagine, glutamine, valine, and isoleucine) and protein levels, and lower nitrate contents, accompanied by a modest sink of organic acids (α-chetoglutarate, succinate, fumarate, and malate), sucrose, fructose, and inulins. Incongruously, the T_7B14 butter heads were less leafy than the controls and showed lowered amino acid/protein contents and overstored inulin. To further investigate the metabolic discrepancies among the GM-lines, a set of key nitrogen and inulin genes was monitored. The T_3B12/T_7B7 lines shared comparable gene expression changes, including the induction of the endogenous *ASPARAGINE SYNTHETASE1* and *NITRATE REDUCTASE1* that supported the targeted enhancement of nitrogen status. Transgene product malfunctioning and T-DNA rearrangements throughout generations were proposed to explain the decreased asparagine content and the complex expression pattern of N genes in T_7B14 leaves. In the latter, the inulin accumulation was associated with the upregulation of fructan biosynthesis genes and the intense repression of fructan hydrolases.

KEYWORDS: Transgenic lettuce (*Lactuca sativa* L.); nitrogen status; NMR metabolic profiling; gene transcription profiling

INTRODUCTION

Lettuce is cultivated worldwide and is economically relevant among the leafy crops (http://www.fao.org). In the omics era, lettuce improvement will benefit the metabolomic scientific approach (*1*) in support of ongoing genomics-assisted programs (e.g.: *Compositae* project http://cgpdb.ucdavis.edu). NMR profiling is a well established technique to picture the degree of metabolic impact induced by plant genetic breeding and bioengineering (*2*). NMR-based surveys, coupled with multivariate analyses, allow one to observe metabolic changes in the transgenic lines monitoring metabolic profiles (*3*−*5*).

Limited leaf nitrate content, crispiness, disease resistance, short cycles, and long shelf life are major targets of lettuce harvesting (*3*). The maximal nitrate levels are under EU restrictions (*4*), and various solutions for NO_3^- management were developed by agricultural techniques (*5*, *6*), conventional breeding (*7*), and biotechnology (*8*). Plant NO_3^- uptake and accumulation are regulated by genetic, environmental, and agricultural factors; among these, N supply and light intensity are crucial for threshold contents. The differing capacities of NO_3^- accumulation also correlate with the diversified location of nitrate reductase (NR) activity and distinct degrees of NO_3^- absorption and transfer (*4*). In vascular plants, N assimilation occurs by NR-mediated conversion of NO_3^- into NH_4^+, which

†This paper is dedicated to the memory of Anna Laura Segre (1938−2008) and Domenico Mariotti (1948−2008), who were brilliant minds in science and sustained the research on genetically modified plants.

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Table 1. Transgenic Lineages

primary transformant[a]	mother plant lineage[b]	progeny name[c]	T-DNA status	*pMAC:asnA* transcript
T_0B12	P08-T_2B12	T_3B12	homozygous	integrum
T_0B7	P32-T_6B7	T_7B7	homozygous	integrum
T_0B14	P31-T_6B14	T_7B14	homozygous	integrum

[a] Primary transformants contained one T-DNA locus; the progenies were selected for the T-DNA homozygous state by scoring phenotype segregation and molecular control. [b] Selected plants (P) produced the seeds generating the progenies for this study. [c] Abbreviated denomination used in this study.

is incorporated into glutamine (Gln) and glutamate (Glu) by the coupled action of glutamine synthetase (GS) and glutamate synthase (*9*). The amino groups of Gln and Glu are used by aspartate amino transferase and asparagine synthetase (AS) to synthesize aspartate (Asp) and asparagine (Asn), respectively. The Gln-dependent AS (Gln-AS, EC 6.3.5.4) is the major source of Asn, which plays a role in regulating N flow [reviewed in ref *10*]. Contrary to plant Gln-AS, prokaryotic asparagine synthetase A of *E. coli* (AS-A, gene *asnA*) uses Asp and NH_4^+ to produce Asn, independently from light (*11*).

Originally, three transgenic lettuce lines for *asnA* (T_0B7, T_0B12,and T_0B14), driven by the chimerical *pMAC* promoter, have been selected since they contained one T-DNA. These lines have shown an increased content of some amino acids, including Asn, and proteins and have been characterized by a better agronomical performance with respect to controls (*12*). Selected phenotypes have been subjected to several rounds of selfing to reach homozygous T-DNA. The descendent progenies T_3B12 and T_7B7 have maintained higher agro-performances than controls, while the Asn contents have not increased in the T_6B14 line though remarkable inulin accumulation, a trait of industrial interest, has occurred at early stages of development (*13*).

This article aims at giving a holistic picture of metabolic and molecular changes associated with the genetic modification targeted to implement Asn content and improve N metabolism in lettuce. In addition, it aims at providing nutritive characteristics of GM versus conventional lettuce at harvesting time, a topic of interest for molecular breeders/biotechnologists and consumers. The NMR spectra combined with statistical analyses allowed us to evidence that, at a given developmental stage, significant changes of amino acids, organic acids, and inulin contents in lettuce genotypes were associated with the transgene; such changes were maximal with respect to controls at a compatible timing with the butter-head harvest (56 DAS). Moreover, the metabolic changes of T_7B14 line (lowered amino acid and augmented inulin contents) were found to be affected opposite to those of the T_3B12/T_7B7 lines that showed N enhanced status. These metabolic discrepancies led us to monitor the transcriptional response of 15 nitrogen and inulin genes (mini-trascriptome) to the transgene. The T_3B12/T_7B7 lines with enhanced Asn content shared a similar mini-trascriptome profile. The gene expression variations consequent to the Asn increase mediated by *pMAC:asnA* add knowledge to the response to the C/N ratio variations in lettuce. The complex transcriptional pattern observed for the overstoring inulin T_7B14 line is discussed in the context that the transgene is constitutively and correctly expressed.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Sampling. Transgenic lines of *L. sativa* cv. Cortina (long-day, butter head, medium green color, and small rosette; NUNHEMS, www.nunhems.com) overexpressing the *E. coli asnA* gene driven by hybrid constitutive *pMAC* promoter (*pMAC:asnA* genotype) were produced by *A. tumefaciens* transformation (*12*). The lineages used in the experiments were the progeny (T_3B12) of selfed plant 8 belonging to the T_2 generation from the transgenic ancestor T_0B12; the progeny (T_7B7) of selfed plant 32 belonging to T_6B7 individuals; the progeny (T_7B14) of plant 31 belonging to the T_6B14 population [see also refs *12* and *13*]; and the progeny from the non-GM Cortina seeds (wild type). All of the individuals sampled were confirmed to produce the integral *asnA* transcript by RT-PCR. The plant lineage synopsis is in Table**1**.

Table 2. Major Agro-Traits of Transgenic and Wild Type Lines

	56 days after sowing			
genotypes	FW (g)[a,c]	leaf number[b,c]	protein content[c] (mg g^{-1} FW)	NO_3^- content[c] (mg kg^{-1} FW)
T_7B14 (P.31)	100.13 ± 7.29[d]	48.23 ± 1.36	1.19 ± 0.08[d]	862.14 ± 62.42[d]
T_7B7 (P.32)	160.32 ± 9.12[d]	58.32 ± 1.49[d]	1.71 ± 0.06[d]	972.05 ± 87.58[d]
T_3B12 (P.8)	157.18 ± 4.83[d]	62.16 ± 1.91[d]	1.83 ± 0.11[d]	748.95 ± 83.45[d]
wild type	123.21 ± 5.87	52.72 ± 2.55	1.54 ± 0.11	1376.91 ± 174.37

[a] FW, fresh weights of heads. [b] Number of expanded leaves; unfolded leaves at the apex were not computed. [c] Mean and standard error were calculated using 15 plants per line. [d] Significant differences (Student's *t* test, $p < 0.05$) of GM versus wild type plants.

Seeds (3 dm^2 per well) were germinated in trays containing soil, peat, and sand into 1:1:1 ratio; seedlings bearing 4−7 leaves were transferred into pots of 10^3 cm^3 and then of 20^3 cm^3 from ca. 25 leaves onward. Each pot was set 5 cm apart from the neighbors. Substrate composition: peat and soil (1:3), and 2 g of fertilizer containing 12% N (5% NH_4+ and 7% NO_3^-) per kg of substrate. Plants were grown in the glasshouse under a natural light/dark cycle at 18/25 °C and watered (pH 7.8, conductivity 0.9−1.3 dsm^{-1}) twice per week. After the first transplant (20/4/2007), we exposed them to one treatment with the insecticide FASTAC (alfametrin) and one with fungicide Rovral FL (iprodione) both by BASF-Crop Protection, Italy.

GM and conventional Cortina plants (sowing date 26/3/2007) were sampled 24, 54, and 64 days after sowing (DAS) at 9:00 a.m. The butter head weights and leafiness are in **Table 2**. At early developmental stages, the growth of all transgenic lines was slightly higher than that of the controls; later on, only T_3B12 and T_7B7 maintained the trait; nonetheless, sampling from the GM and control plants ($n = 15$ for each genotype and at each growth stage) occurred in the same morning. At 24 DAS, whole aerial parts of seedlings were sampled; at 56 and 64 DAS, fully expanded leaves ($n = 7$ per plant, from the 28th to the 35th leaf) borne in the middle of the rosette were pooled from each plant. Regarding NMR analyses, leaves ($n = 3$) were cut at the petiole, transferred to boron-silicate glass vials (for seedlings) or large neck flasks (for adult plants), immediately frozen with liquid nitrogen, transferred to a −30 °C precooled apparatus, and lyophilized for 24 up to 48 h, depending on the amount of sampled material. In general, 500 mg of fresh weight produced ca. 30 ± 0.87 mg of lyophilized material (ca. 6% yield). As for molecular analyses, the pool per plant (leaves $n = 2$) was frozen by liquid N and stored at −80 °C; as for nitrate contents, the pool per plant (leaves $n = 2$) was dehydrated at 80 °C for 72 h. Only samples at 56 DAS were used for the latter two analyses.

Extractions of Water and Organic Soluble Compounds. Excised tissues were immediately lyophilized for 48 h, stored at −20 °C, and aliquots for experiments were ground to powder in liquid nitrogen. The lipid fraction was isolated according to refs *14* and *15*. Briefly, powdered tissues (25 mg) were homogenized in 1 mL of 0.15 M acetic acid for 2 min, then 10 mL of a chloroform/methanol mixture (1:1 v/v) was added, the suspension was stirred for 2 min at (4 °C), and then 2 mL of distilled water was added. Phase separation was obtained by centrifugation at 9000*g* for 20 min (4 °C); the chloroform layer and aqueous fraction were separated. The aqueous fraction was discarded, while the chloroform fraction was clarified by filtration through cotton wool, fully evaporated under N_2, and finally stored at −20 °C. The residue was dissolved in a $CD_3OD/CDCl_3$ mixture (3:2 v/v) at RT and transferred into a standard 5-mm NMR tube. As for the water-soluble fractions, the methods in refs *14* and *15* allow the separation of the aqueous fractions from the organic fraction; however, we have experienced that acetic acid residues may affect the spectrum quality. Therefore, the following method was used: ground tissues (21 mg) were mixed with 1 mL of a D_2O phosphate buffer for 2 min at RT (Na_2HPO_4/

NaH_2PO_4, 400 mM salt concentration, 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS), 50 μM EDTA, and pD value of 6). EDTA was added to prevent the broadening of the ^{1}H NMR signals of citric and malic acids due to paramagnetic cations. In the case of investigated samples, ^{1}H NMR signals of EDTA were not overlapped with the resonances of analyzed metabolites. The mixture was centrifuged at 9000*g* for 7 min; the supernatant was filtered and transferred in a standard 5 mm NMR tube.

NMR Spectroscopy. 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 probe head capable of producing gradients in the *z* direction with a strength of 55 G cm^{-1}. Spectra were assigned using literature data (*16*).

^{1}H NMR Spectra of Aqueous Extracts. Proton spectra were referenced to the signal of DSS methyl groups at $\delta = 0.00$ in D_2O. ^{1}H spectra of aqueous extracts were acquired by coadding 512 transients with a recycle delay of 2.5 s and 32 K data points. The residual HDO signal was suppressed using presaturation with a long single soft pulse applied during relaxation delay. To avoid possible saturation effects on unknown compounds, the experiment was carried out by using a 45° flip angle pulse of 8.0 μs. The intensity of 24 ^{1}H resonances due to water-soluble assigned metabolites were measured with respect to the intensity of the DSS signal (0.1 mM) normalized to 100 used as the internal standard. The 24 measured resonances, see **Figure 1** and the list of abbreviations, are due to Ile (**1**, 1.00 ppm), Val (**2**, 1.02 ppm), LA (**3**, 1.31 ppm), Thr (**4**, 1.33 ppm), X (**5**, 1.35), Ala (**6**, 1.48 ppm), Glu (**7**, 2.05 ppm), GABA (**8**, 2.29 ppm), MA (**9**, 2.36 ppm), SA (**10**, 2.40 ppm), Gln (**11**, 2.47 ppm), CA (**12**, 2.51 ppm), Asp (**13**, 2.82 ppm,), Asn (**14**, 2.88 ppm), α-KGA (**15**, 2.99 ppm), CHN (**16**, 3.19 ppm), *β*-Glc (**17**, 3.22 ppm), INOS (**18,** 3.27 ppm), Fruct (**19**, 4.00 ppm), TA (**20**, 4.32 ppm), Sucr (**21**, 5.41 ppm), Inul I (**22**, 5.42 ppm), Inul II (**23**, 5.43 ppm), and FA (**24**, 6.51 ppm).

^{1}H NMR Spectra of Organic Extracts. Proton spectra were referenced to the CH_3 resonance of TMS in $CD_3OD/CDCl_3$. ^{1}H spectra of organic extracts were obtained using the following parameters: 256 transients, 32 K data points, recycle delay of 3 s, and a 90° flip angle pulse of 10 μs.

The integrals of 11 ^{1}H resonances due to assigned liposoluble metabolites (*16*) were measured and used to obtain the normalized integrals. The resonances are due to CH_3-18 of *β*-sitosterol (**1′**, 0.68 ppm), CH_3-18 of stigmasterol (**2′**, 0.70 ppm), CH_2 allylic protons (**3′**, 2.07), CH_2-2 of free fatty chains (**4′**, 2.29 ppm), CH_2-2 of esterified fatty acids in triglycerides, phospholipids, galactolipids, and sulpholipids (**5′**, 2.33), CH_2-11 of linoleic acid (**6′**, 2.77 ppm), CH_2-11,14 of linolenic acid (**7′**, 2.80 ppm), CH-1′ of sulpholipids (**8′**, 4.80 ppm), CH-1′′ of galactolipids (**9′**, 4.90 ppm), CH-double bound proton of squalene (**10′**, 5.11 ppm), and CH-double bound proton of carotenoids (**11′**, 6.62 ppm). The normalized integral of a specific metabolite corresponds to its relative molar concentration with respect to all (free and esterified) fatty acid chains, regarded as 100%, and can be calculated according to the following eqs 1−3. Taking into account that the integral of an ^{1}H NMR signal is proportional to the number of protons with the same chemical shift, the coefficients 67, 100, or 200 were used for CH_3, CH_2, and CH, respectively.

$$nI_X = (I_X/(I_{4'} + I_{5'})) \times 67, \text{ if } X = CH_3 \quad (1)$$

$$nI_X = (I_X/(I_{4'} + I_{5'})) \times 100, \text{ if } X = CH_2 \quad (2)$$

$$nI_X = (I_X/(I_{4'} + I_{5'})) \times 200, \text{ if } X = CH \quad (3)$$

where nI_X is the normalized integral of the X signal, I_X is the integral of the X signal, and $I_{4'} + I_{5'}$ is the sum of the integrals of all fatty acid CH_2-2 signals.

Statistical Methods for NMR Data. Signal intensities and integrals of selected resonances were submitted to a suitable statistical analysis by using the Statistica software package for Windows (1997; edition by Statsoft). The ANOVA 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

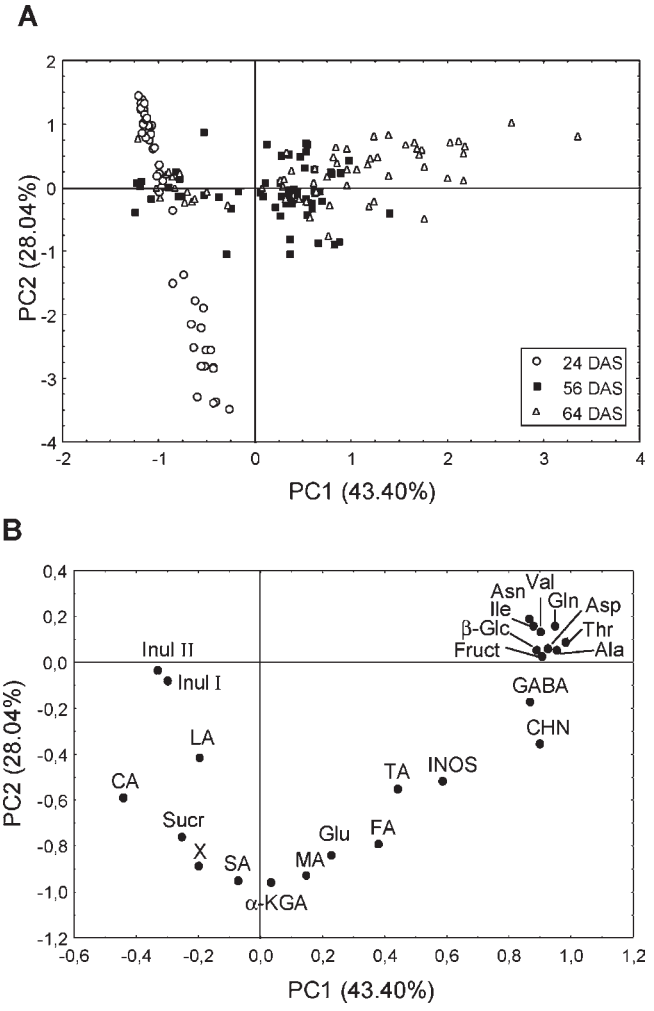

Figure 1. PCA based on hydro-soluble metabolites. (**A**) PC2 versus PC1 scores after variables loadings of 59 transgenic and control leaf samples at 24 days after sowing (DAS) (○), 60 samples at 56 DAS (■), and 61 samples at 64 DAS (Δ). (**B**) PC2 versus PC1 scores including the 24 hydro-soluble compounds.

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 therefore drastically reduces the number of variables to be analyzed (*17*). Before performing PCA, all variables were mean-centered, and each variable was divided by its standard deviation (autoscaling). Linear discriminant analysis (LDA) is a multivariate method used to determine which variables discriminate better between two or more *a priori* defined groups. This procedure needs selected variables to build up a data matrix and to give rise to discriminant (canonical) linear functions. The separation degree among the groups at each stage was calculated (Table S1 of Supporting Information) by the squared Mahalanobis distance (SMD), the probability that a sample belongs to a particular group is proportional to the SMD from the group's centroid; high values of SMD between the centroids of two groups imply a significant separation grade (*17*).

Real Time RT-PCR (qRT-PCR). The frozen leaf pool per plant (leaves $n = 2$) was ground, and the powder was used for both gene expression analysis and SST enzymatic assays. Total RNA was isolated with TRIzol reagent (INVITROGEN), DNase-treated (RQ1, PROMEGA), and quantified by the spectrophotometer (NanoDrop technologies), and 3 μg was reverse transcribed at 55 °C into a single strand cDNA by oligo-$(dT)_{20}$ by Superscript III, according to the manufacturer's instructions (INVITROGEN). Table S2 of the Supporting Information contains details on gene encoded proteins, primers, PCR conditions, and gene

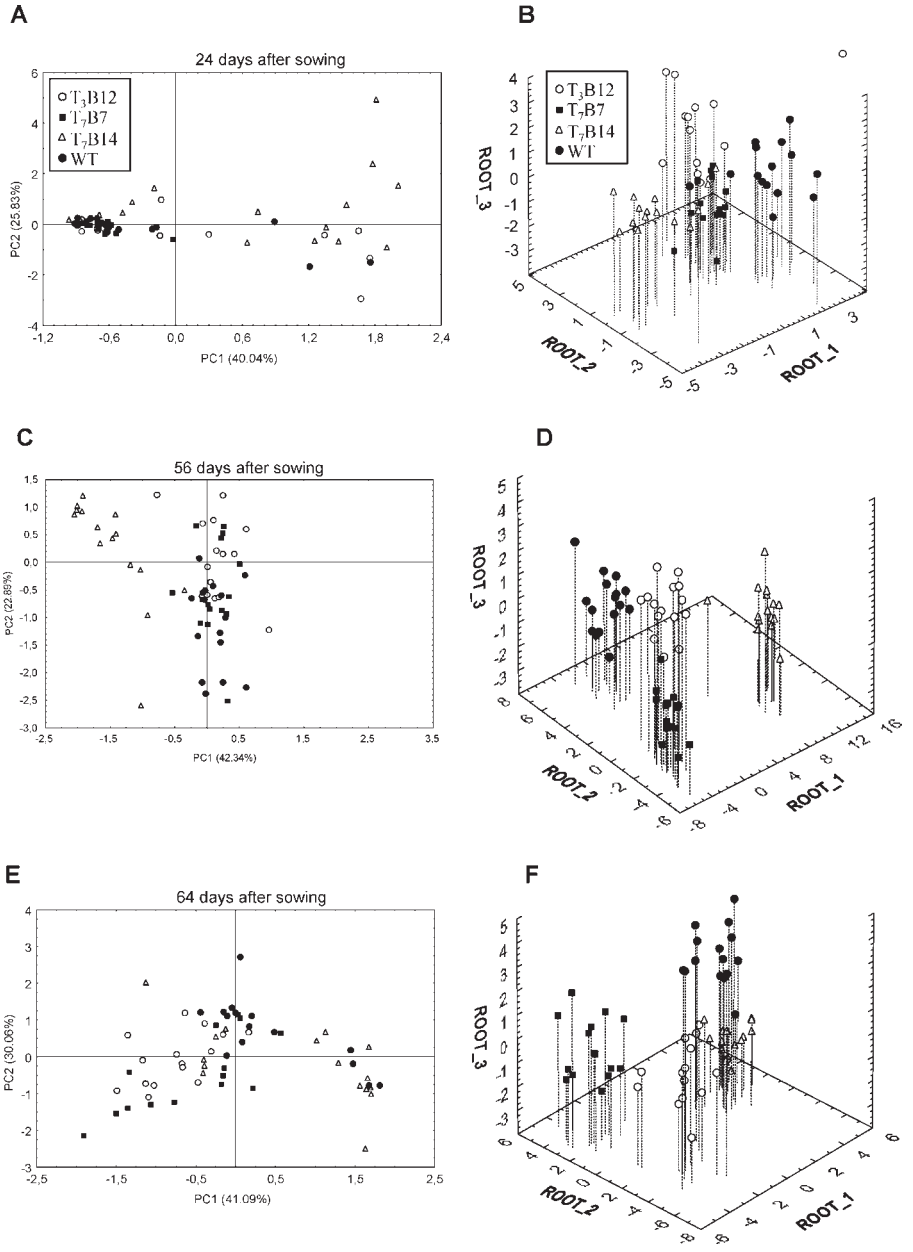

Figure 2. PCA (**A**, **C**, and **E**) and LDA (**B**, **D**, and **F**) of transgenic and control lettuce samples at 24, 56, and 64 days after sowing. The genotypes are indicated by symbols in **A** and **B**, and consistent with those of the other panels.

expression levels. The cDNA (40 ng) was amplified in 20 μL of total volume containing the 1X reaction buffer; 50 μM dNTPs; 3.5 mM $MgCl_2$; 0.6 M D-(+)-trehalose dihydrate (Fluka); 0.7 M formamide; 0.1 μL of a 1:100 Sybr Green I diluition in dimethylsulfoxide (INVITROGEN); 0.2 U Taq DNA polymerase (QIAGEN); and 0.3 μM of each primer. Conditions: 180 s at 95 °C and 45 cycles of three-step amplification (30 s at 95 °C, 1 min at 58 °C, and 1 min at 72 °C). The threshold cycle (C_t) value of each gene was normalized with that of *ACTIN2* and compared with the C_t of an untreated control using the $2^{-\Delta\Delta Ct}$ method (*18*). The experiments were in biological and technical triplicates. The diagrams of relative mRNA abundance represent actin-normalized values of GM samples with respect to those of controls.

Nitrate Contents. The nitrates were isolated from 0.5 g of dried material and measured by ionic chromatography (Dionex DX 250, Dionex Corp, Sunyvale, CA) with AS4A columns as described in ref *5*.

Protein Content and SST Assays. The same material as that of gene expression experiments was used. The total soluble protein concentration was determined by the dye-binding method of Bradford using BSA as the standard (*19*). The leaf SST activity was assayed according to ref *20*.

RESULTS AND DISCUSSION

Profiles of Transgenic and Control Samples. Typical ^{1}H NMR spectra of water-soluble and organic fractions were produced together with the spectral assignment (Supporting Information) of the resonances (*16*) . The ^{1}H spectra of the four genotypes show the same signals indicating that spectra of transgenic and control leaves were conservative, although signal intensity varied with the growth stages and genotype. The NMR-statistical protocol was based on the measurement of the selected signal intensity, which was processed by multivariate statistics. All samples derived from both GM and control lines at three growth stages (24, 56, and 64 DAS) were analyzed, and the signal intensity was submitted to the statistical analysis. The sample grouping and variables with discriminant power were determined by the PCA, LDA, and ANOVA methods.

As for hydro-soluble compounds, the PCA was performed on 180 samples (**Figure 1A**); the combined PC1 and PC2 explained 71.44% of total variance and together were able to separate the

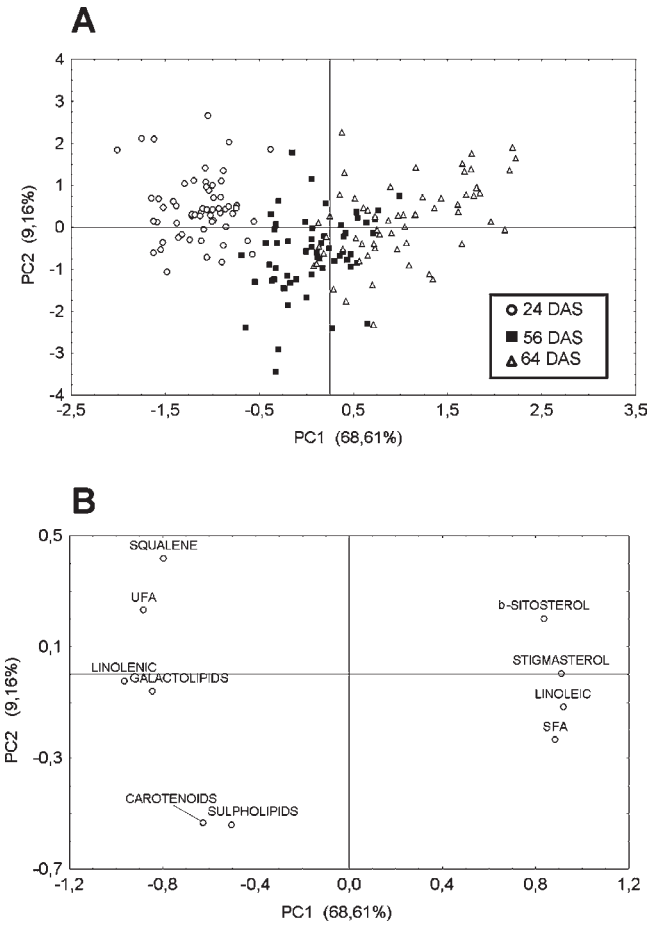

Figure 3. PCA based on 24 organic metabolites: (**A**) PC2 versus PC1 scores and (**B**) variable loadings of 59 transgenic and control leaf samples at 24 DAS (○), 59 samples at 56 DAS (■), and 61 samples at 64 DAS (Δ).

three developmental stages. Amino acids Ile, Asp, Asn, Gln, Val, Thr, Ala, GABA, sugars (fructose and β-glucose), and choline had the highest discriminant power as evidenced in the loadings plot (**Figure 1B**). The global PCA clearly shows sample groupings according to the sampling time. The latter corresponds to the diversified physiological status of leaf development (maturation) and appeared to be mostly responsible for compound variability. Hence, assuming that variations depending on the environment had been minimized, the maturation process was likely to be the major cause of metabolic changes in lettuce leaves. To some extent, this is consistent with previous results on tomato berry, which showed that physiological changes of organ development per se accounted for the variation of metabolic profiles in tomato berry ripening (*21*).

In order to avoid the masking of the contribution of genetic modifications to variability, PCA (**Figure 2A**, **C**, and **E**) and LDA (**Figure 2B**, **D**, and **F**) were performed separately for each developmental stage. PCA and LDA indicated that the most defined clustering of genotypes occurred at 56 DAS (**Figure 2C** and **D**). The SMD further confirmed that the highest separation degree (Supporting Information) between the conventional versus the GM-lines occurred at 56 DAS. The SMD values (T_7B14 = 161.58; T_7B7 = 83.53; T_3B12 = 54.83) indicated that the T_7B14 genotype was the most distant from the controls, followed by the T_7B7 and by T_3B12 lines. Taken together, these analyses showed the association between significant metabolic variations and the GM genotypes at a given stage, suggesting that transgenesis contributed to the variance of hydro-soluble compounds.

Table 3. Fold Change of Metabolite Molecular Abundance in Transgenic Lines Compared to That of Controls at Harvesting

	fold changes[a] at 56 DAS					
metabolites	T_3B12		T_7B7		T_7B14	
amino acids	mean	s.e.	mean	s.e.	mean	s.e.
Ala	1.12	±0.10	1.08	±0.08	**−2.93**	**± 0.37**
Val	**1.42**	**± 0.16**	**1.42**	**± 0.12**	**−2.93**	**± 0.42**
Glu	−1.14	±0.11	−1.08	±0.12	**−2.53**	**± 0.27**
Gln	**1.53**	**± 0.18**	**1.44**	**± 0.18**	**−6.58**	**± 2.19**
Asp	1.03	±0.12	1.09	±0.10	**−3.01**	**± 0.51**
Asn	**2.10**	**± 0.31**	**2.12**	**± 0.31**	**−6.90**	**± 2.03**
Thr	1.08	±0.09	1.07	±0.08	**−3.87**	**± 0.67**
Ile	**1.36**	**± 0.16**	**1.34**	**± 0.13**	**−2.65**	**± 0.42**
GABA	1.16	±0.10	1.13	±0.11	**−2.56**	**± 0.32**
KCOA	mean	s.e.	mean	s.e.	mean	s.e.
CA	1.27	±0.15	1.12	±0.13	**1.80**	**± 0.32**
α-KGA	**−1.36**	**± 0.13**	−1.06	±0.09	−1.07	±0.12
SA	**−1.33**	**± 0.12**	−1.03	±0.10	−1.22	±0.14
FA	**−1.72**	**± 0.20**	**−1.52**	**± 0.17**	**−2.95**	**± 0.50**
MA	**−1.27**	**± 0.08**	−1.08	±0.06	**−1.30**	**± 0.07**
sugar	mean	s.e.	mean	s.e.	mean	s.e.
β-Gluc	−1.18	±0.11	−1.19	±0.10	**−1.76**	**± 0.28**
Fruct	−1.16	±0.09	−1.09	±0.08	**−2.14**	**± 0.36**
Sucr	−1.14	±0.15	**−1.34**	**± 0.18**	**3.10**	**± 0.32**
Inul I	**−1.70**	**± 0.32**	**−3.04**	**± 0.55**	**19.02**	**± 3.28**
Inul II	**−1.50**	**± 0.31**	**−2.02**	**± 0.39**	**69.11**	**± 13.45**

[a] Positive values represent the T/C ratio, and the negative values are −C/T. T, transgenics; C, control.

As for the organic fraction, PCA applied to 179 samples (**Figure 3A**) showed that the values of PC1 (68.61%) and PC2 (9.16%) accounted for 77.77% of the total variance. PC1 mainly contributed to separate the developmental stages, and the most discriminant variables were stigmasterol, β-sitosterol, linoleic, linolenic, and saturated fatty acids (**Figure 3B**). The analyses suggest that leaf maturation status is the major factor responsible for the changes in the liposoluble molecules, similar to the effects observed on water-soluble compounds. However, PCA and LDA at each growth stage did not reveal any significant separation among the four genotypes (data not shown). Therefore, transgene contribution to liposoluble compound variance could not be evidenced using the selected compounds.

Traits of *pMAC:asnA* and Conventional Butter Heads at Harvesting. As reported above, the highest separation degree between the conventional versus the GM-lines occurred at 56 DAS, which coincided with the butter head optimal harvest with respect to the Italian discipline on lettuce from protected cultures (minimum head weight, 100 g; NO_3^- content, 2.5 g·kg^{-1} fresh mass). At 56 DAS, T_3B12 and T_7B7 rosettes were more leafy and heavier than the controls and had higher leaf protein content (**Table 2**); while the T_7B14 heads weighed slightly less than the controls, leafiness did not vary, and protein content was reduced. Interestingly, the NO_3^- contents of all GM-lines were significantly lower than those of the wild type. The molecular abundance fold changes of hydro-soluble compounds with discriminant power (aa, KCOA, and sugars) in GM versus control lines are summarized in **Table 3** (significant variations are bold faced). Overall, T_3B12 and T_7B7 shared similar metabolic features, while that of T_7B14 was skewed from the other two. Hence, both results and the discussion refer commonly to T_3B12 and T_7B7 and specifically to T_7B14.

T_3B12 and T_7B7 Lines Undergo N Status Enhancement. As for the leaf N status in the case of T_3B12 and T_7B7, some aa, mainly

Asn and Gln, were ca. 2-fold higher than the controls (**Table 3**), while Glu showed unvaried levels. Both lines maintained enhanced growth and content of aa and total protein (**Table 2**) at maturity due to the expected *pMAC:asnA* effect, consistent with previous data (*12*). The Glu steady state may reflect the constant and quick turnover of this branch-point amino acid, which is recruited in several plant pathways (*22*). The overexpression of *asparagine synthetase* of plant (*23*, *24*) and prokaryotic origin (*12*, *25*−*27*) affects the N status of transgenic plants, producing opposite or unpredicted effects on the aa contents, which were explained by various reasons including the crop specific genetic background, the complexity of the N regulatory pathway in diverse species, the transgene correct functioning, and gene silencing phenomena.

Concerning the C status, both GM lines showed an overall diminution of KCOA and simple and complex sugars. Independent of the causes, the NMR metabolic profile showed that the C/N equilibrium was altered in both lines, suggesting that carbon skeletons were recruited for aa biosynthesis rather than sugar storage. For instance, the skeleton for Glu and Gln comes from α-KGA, which drops in GM plants; similarly, the decrease of FA, SA, and MA may reflect the consumption of oxalacetate (not measured by NMR), which is the C source for Asp and Asn syntheses. The diminished nitrate levels of GM-leaves together with the increased levels of total proteins and target amino acids further supported this hypothesis (**Table 2**). This profile prompted us to monitor the leaf expression of a gene set related to N and inul pathways; the gene number depended on the availability of lettuce EST sequences in current databases and on the primer effectiveness in qRT-PCR experiments. Supporting Information reports the relative mRNA abundance of all tested genes; only those with significant variation with respect to controls were graphed (**Figure 4**). As for the amino acid metabolism (**Figure 4A**), one of the three Gln dependent *AS* genes (*AS1*) was up-regulated, while the *GS* transcription was repressed compared to that of controls. Regarding the nitrate pathway, the *NR1* and *NRT1* mRNA abundances of both GM lines were higher than those of the controls.

Prior to discussion, a mandatory premise regarding the regulation of N metabolism (NM) genes needs to be mentioned. Nitrate assimilation and aa homeostasis involve a variety of control mechanisms; both of them share complex interactions with other leaf cell pathways (e.g., photosynthesis and organic acid cycles). Moreover, most NM genes form families; the members undergo distinct control processes (at the transcriptional, post-transcriptional, and post-translational levels) driven by light (circadian rhythm, photoperiod, and intensity), N and C metabolites, and C/N ratios (*9*, *28*). Finally, root NM also plays an important role in the regulation of N flux to leaves (*29*); in this work, NM gene transcript profiling was targeted to lettuce leaves (agro-product), regardless of *pMAC:asnA* effects in roots and possible cross-talk with leaf metabolism.

Most plant *AS* genes commonly share the upregulation in response to both increased supply of NH_4^+ and aa, and to soluble carbohydrates reduction (*10*). Moreover, the *Arabidopsis AS* members (*ASN1* and *ASN2*) are differentially and reciprocally regulated by light, C, and N metabolites (*30*). Overall, the organic C/N ratio is considered as the final modulator of *ASN1* expression. In particular, Asn supply can compensate sugar and light repression effects on *ASN1* (*31*), which is normally down-regulated by daylight. In this context, the *AS1* 3-fold induction in morning-sampled lettuce leaves (see Materials and Methods) suggested that the *pMAC:asnA* driven production of Asn induced lettuce *AS1*, which is consistent with the fact that Asn supply can antagonize the light repressive effects on *Arabidopsis ASN1*

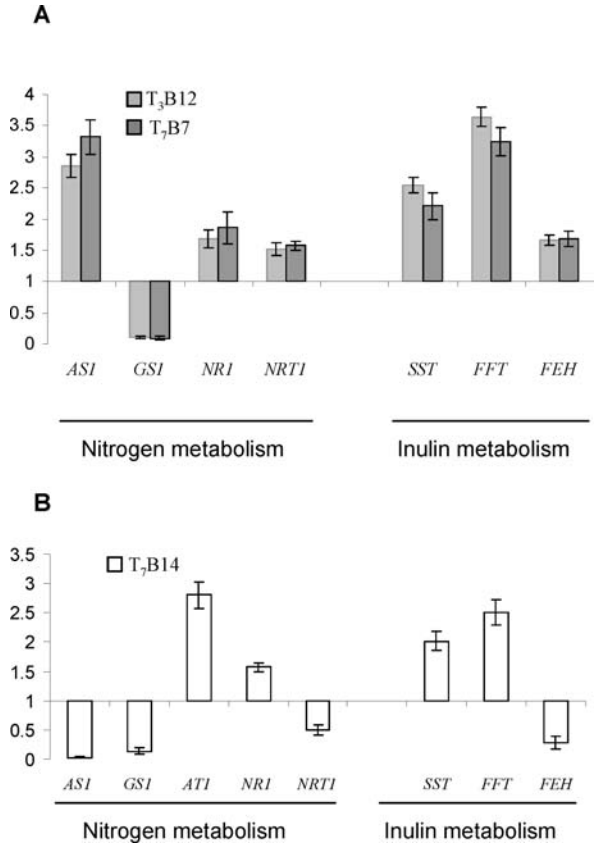

Figure 4. Expression patterns of N and inulin genes in leaves at 54 DAS. The relative mRNA abundance of genes involved in N and inulin metabolism was monitored by q-RT-PCR in both transgenics and controls (see also Table S2 in Supporting Information). The figure reports the fold change transcript abundance of selected genes having significant variation with respect to controls. The gene transcription profile in T_3B12 and T_7B7 (**panel A**), and in T_7B14 (**panel B**). The *ASPARAGINE* and *GLUTAMINE SYNTHASE* (*AS1*, *GS*), and *AMINO ACID TRANSPORTER* (*AT1*) genes fall into aa synthesis and mobilization; the *NITRATE REDUCTASE* (*NR*) and high affinity *NITRATE TRANSPORTER* (*NRT1*) genes belong to the NO_3^- organication pathway; and the *SUCROSE:SUCROSE 1-FRUCTOSYL TRANSFERASE* (*SST*), *FRUCTAN:FRUCTAN 1-FRUCTOSYL TRANSFERASE* (*FFT*), and *FRUCTAN EXOHYDROLASE* (*FEH*) genes take part in inulin biosynthesis and catabolism. Standard deviation for ratios was calculated and represented by bars. All data sets were subjected to Student's *t* test where $p < 0.05$ was regarded as significant.

transcription (*31*). The response of lettuce *AS1* to Asn synthesis may further push the store of inorganic N into organic reserves, as suggested by the depletion of KCOA and sugars. Finally, the regulation of other lettuce *AS* members needs further surveys to interpret the unvaried expression of *AS2* and *AS3* (Supporting Information).

Several *AS* overexpressing plants, including the T_3B12 and T_7B7 lines, show an increase of Gln contents in leaves (*23*, *26*, *27*). Transcription of genes encoding cytosolic GS is under different modes of regulation (*32*). In general, the organic C/N ratio ultimately regulates *GS* genes; specifically, *GS* genes are abundantly transcribed under the light, triggered by N inorganic nutrients and repressed by Asn and Gln supplies. The lettuce cytosolic *GS* mRNA drop may be consequent to the direct Asn augmented levels. Moreover, the rise in Gln in GM leaves may be a result of (a) GS enzymatic activity, which is known not to follow the respective gene expression (*32*), and (b) the action of other *GS* members, which was not tested since only one sequence was available.

Table 4. Sucrose: Sucrose 1-Fructosyltransferase (1-SST) Activity in *pMAC:asnA* and Control Genotypes at 56 Days after Sowing

genotype	1-SST activity (nanomol thrisaccaride × mg prot^{-1})
wild type	68.97 ± 0.79[a]
T_3B12	71.76 ± 0.85[b]
T_7B7	78.78 ± 0.52[b]
T_7B14	102.21 ± 0.68[b]

[a] Standard deviation. [b] Significant by Student's *t* test versus the wild type ($p < 0.05$).

Nitrate enters leaves mainly via the xylem (*33*); consequently, it is either reduced to NH_4^+ (by the nitrate reductases, NRs) and directed to chloroplasts or stored as such into vacuoles, from which it is remobilized toward other plant organs (by the nitrate transporters, NRTs). The *NRs* (*34*) and *NRTs* (*35*) genes are under complex control mechanisms. The *NR* transcript and enzyme activity vary diurnally, and the downstream products (e.g., Gln, Asn) are thought to downregulate *NR* expression by unclear feedback mechanisms. However, both *NR*s and low affinity *NRTs* (*NRT1s*) genes are induced by NO_3^-, while the inhibition by Glu and NH_4^+ supply is reported for *NR* [with controversial data (*9*)] and assessed only for high affinity *NRTs* (*NRT2s*). The diurnal upregulation of *NR1* and *NRT1* in T_3B12 and T_7B7 leaves compared to that in controls (**Figure 4A**) suggests that these genes perceive the aa content alterations. The exogenous prokaryotic AS-A enhances the Asn content competing with other enzymes in the recruitment of NH_4^+ (e.g., from *NR* action, photorespiration, and aa catalysis) and Glu. Gradients of NH_4^+ concentration might stimulate NO_3^- mobilization exerting feed forward effects on *NR1* and *NRT1* transcription. Eventually, the lower nitrate contents of GM leaves may be explained by the increase of NR and NRT1 activities, which are known to go in parallel with the respective gene transcription (*36*, *37*).

Referring to inulin homeostasis, the expression of the biosynthetic *FFT* and *SST* and the catabolic *FEH* genes was higher than that of the controls (**Figure 4A**), suggesting that the global pathway was enhanced. This pattern was accompanied by a slightly higher activity of the SST enzyme (**Table 4**). Most fructan genes are controlled transcriptionally by light and sugars, and the expression levels go in parallel with protein abundance and enzyme activity. The role of N metabolites on inulin gene transcription is much less known (*38*, *39*). The decrease of KCOA and sugar values in T_3B12 and T_7B7 lines suggested that the organic C was consumed more intensely in GM than in control leaves. Therefore, inulin decrease might be a consequence of catabolic events, as supported by *FEH* upregulation, rather than synthesis inhibition. In other words, inulin remobilization may prevail to provide C back bones for the aa synthesis prompted by the transgene.

T_7B14 Heads Accumulate Inulin. At 56 DAS, the T_7B14 lettuce heads weighed slightly less than the controls, and leafiness was not significantly different (**Table 2**). As for N compounds, the total protein and nitrate contents of T_7B14 leaves diminished, together with an intense decrease of aa levels with a striking drop of Asn and Gln (**Tables 2** and **3**). Concerning C metabolites, the KCOA, β-Glc, and Fruct of transgenic leaves decreased, whereas both Sucr rise and Inul accumulation were observed (**Table 3**). These parameters suggested the occurrence of C/N status alteration in which the KCOA, β-Glc, and Fruct consumption was directed toward C metabolism rather than the amino acid pathway. The T_7B14 metabolic skewing from the T_3B12/T_7B7 lines was also observed in the expression patterns of N and Inul genes (**Figure 4B**). As for the aa pathway, the *AS1* and *GS1* were both repressed, while the *aminoacid transporter* (*AT1*) gene was induced; as for the NO_3^- metabolism, *NR1* was triggered in opposition to the behavior of *NRT1*.

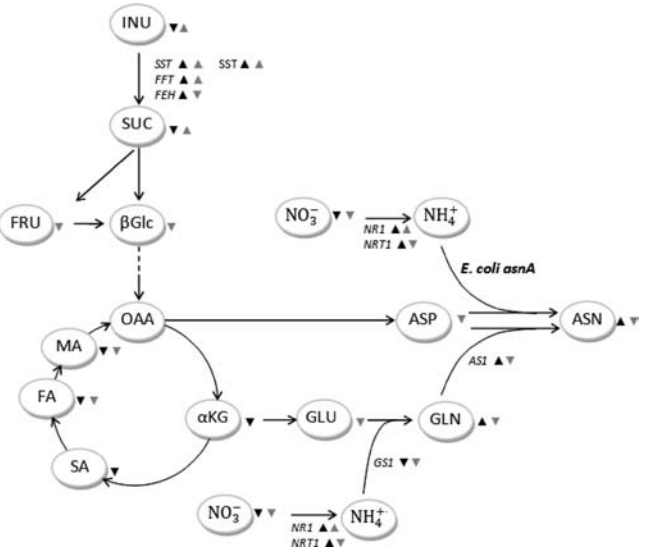

Figure 5. Overview of changes in *pMAC:asnA* lettuce lines. Metabolites are encircled, and endogenous plant genes and enzyme (SST) are italicized and in normal font, respectively. The additional synthesis of Asn mediated by the transgene (*pMAC:asnA*) is bold faced. The increase (upward arrowheads) and decrease (downward arrowheads) of metabolite contents, mRNA levels, and enzyme activity refer to T_3B12/T_7B7 (black arrowheads) and T_7B14 (gray arrowheads) with respect to control genotypes. Unvaried levels are not reported (see also **Tables 3** and **4** and Supporting Information). The acronym expansions are in the Abbreviations Used section.

The unexpected Asn drop suggests the occurrence of *pMAC:asnA* malfunctioning; however, no significant variations of both transgene expression and protein levels were measured among the three GM lines at a given sampling time or in different leaf types (data not shown). Moreover, transcript sequence controls did not reveal mRNA mutations; hence, we may speculate that AS-A activity varied due to protein modification/editing events, which were not surveyed in this work. In addition, AS activity determination is hampered by major factors such as enzyme instability, AS inhibitors, and asparaginase activity that contaminate plant extracts (*12*). Another hypothesis evokes the occurrence of T-DNA rearrangements and/or meiotic recombination observed for transgenes that retain expression (*40*). We previously proposed that the discrepancies between T_2B14 and T_6B14 at 21 DAS were caused by different seasonal light conditions (sampling in late May versus April) that acted on the N metabolism of the long day variety Cortina (*13*). In this work, metabolic spectra of T_6B14 (*13*) and T_7B14 leaves at 24 DAS (both genotypes were sampled in April) were substantially coherent in terms of aa and inulin variations (not shown), suggesting that the T-DNA impact was stabilized. Considering that the lettuce genetic background is highly and stably homozygous (*3*) and that the *pMAC:asnA* expression was unpaired, we speculate that events of T-DNA rearrangements might have occurred at early generations and have caused interferences with the endogenous genes that were subsequently maintained. The rescue of the T-DNA flanking regions may be useful to assess whether and which adjacent genes have been affected; however, this issue was beyond the aim of the work considering that the lettuce genome has not been sequenced yet. In this scenario, the N gene responses of T_7B14 leaves require complex interpretation, which is feebly supported by the literature. The *AS* and *GS* downregulation supports the aa content decrease (**Table 3**), while the *AT1* high induction suggests that the aa depletion is enhanced by mobilization. Moreover, the *NR1*

induction is consistent with lowered nitrate content (**Table 2**), and the *NRT1* downregulation suggests a reduced transport of ammonium, further consistent with diminished levels of N transporter aminoacids (Asn and Gln).

As for the inulin genes, the T_7B14 and T_3B12/T_7B7 plants shared the upregulation of *FFT* and *SST* (compare **Figure 4A** and **B**) and raised SST activity (**Table 4**); T_7B14 had the highest values versus those of the controls (**Table 4**), confirming that *SST* mRNA upregulation was associated with enzyme increase and activity (*41*, *42*). Contrary to T_3B12/T_7B7, the *FEH* gene was significantly downregulated in T_7B14 leaves (compare **Figure 4A** and **B**), suggesting that inulin remobilization may be either hampered or relented in favor of accumulation.

In conclusion, the NMR profiles provided a wide picture of nutritional features of GM-lettuce at harvesting. Statistical analyses of NMR data indicated the metabolic variations associated to *pMAC:asnA* transgene at a given developmental stage and showed that the T_7B14 significantly differed from T_3B12/T_7B$_7$ lines. The enhanced N status of the latter is likely to be a consequence of the Asn increment mediated by the proper transgene function, which alters the transcription of key lettuce N genes (**Figure 5**), eventually leading to protein increase and nitrate diminution at moderate expenses of sugar consumption. Contrary to the T_3B12/T_7B7 lines, T-DNA rearrangements and transgene malfunction at the level of AS-A enzyme activity were speculated to underlie the impairment of N metabolism/gene transcription and the accumulation of inulin (**Figure 5**). Such storage is likely to be a result of a decreased catabolism/mobilization of fructans and represents a trait of agronomical and industrial interest.

ABBREVIATIONS USED

Ala, alanine; Asn, asparagine; Asp, aspartic acid; CHN, choline; CA, citric acid; DAS, days after sowing; Fruct, fructose; FA, fumaric acid; GABA, γ-aminobutyric acid; Gln, glutamine; Glu, glutamic acid; β-Glc, β-glucose; Inul I, inulin I; Inul II, inulin II; Ile, isoleucine; α-KGA, α-ketoglutaric acid; LA, lactic acid, MA, malic acid; INOS, myo-inositol; SFA, saturated fatty acids; SA, succinic acid; Sucr, sucrose; TA, tartaric acid; UFA, unsaturated fatty acids; Thr, threonine; X, methyl group of an unknown compound; Val, valine; KCOA, Krebs' cycle organic acids; aa, amino acid; AS1, asparagines synthetase 1; GS, glutamine synthetase; AT1, amino acid transporter; NR, nitrate reductase; NRT1, (high affinity) nitrate transporter; SST, sucrose:sucrose 1-fructosyl transferase; FFT, fructan:fructan 1-fructosyl transferase; FEH, fructan exohydrolase.

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Supporting Information Available: Typical ^{1}H NMR spectra of water soluble lettuce leaves metabolites from a control sample at 56 DAS at 300 K; typical ^{1}H NMR spectra of the organic fraction of lettuce metabolites from a control sample at 56 DAS at 300 K; water soluble and organic ^{1}H NMR spectral data; squared Mahalanobis distances; and lettuce gene information. This material is available free of charge via the Internet at http://pubs.acs.org

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