Chapter

Metabolic Profiling of Transgenic Tobacco Plants Synthesizing Bovine Interferon-Gamma

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Abstract

Interferon-gamma belongs to a large family of cytokines – multifunctional secreted proteins involved in animal non-specific immune response. Previously inbred lines of *Nicotiana tabacum* L. plants harboring a heterologous gene of bovine interferon-gamma *Bt-sIFNG* under the control of a constitutive 35S CaMV promoter have been created by *Agrobacterium*-mediated genetic transformation. The antiviral and immunomodulatory activities of plant-produced interferon-gamma in bovine cell culture and laboratory animals (mice) were observed. A state-of-the-art GS-MS technique has been used to identify the possible effect of the transformation on the plant's metabolome. Total profiles included 350 metabolites from leaves, among which 150 substances were identified up to their class and 80 up to the exact metabolite. Metabolite profiling revealed that plants able to synthesize interferon-gamma are characterized by a higher level of amino acids and other substances involved in nitrogen metabolism. In transgenic plants intensification of the secondary metabolism was also detected. Some alterations were distinguished in plant metabolome depending on cultivation conditions.

Keywords: interferon-gamma, *Nicotiana tabacum*, transgenic plant, metabolome

1. Introduction

Metabolic alterations triggered by biotic and abiotic factors in the environment are the basis of a plant development and adaptation. They are closely related to the shifts in primary as well as secondary metabolism that are responsible for synthesis and accumulation of different regulatory and defensive metabolites [1–3]. Metabolic profiling is a useful tool for distinguishing these alterations. Powerful approaches to provide metabolomic investigations are based on recent technology such as GC–MS (gas chromatography–mass spectrometry), LC–MS (liquid chromatography–MS), CE–MS (capillary electrophoresis–MS), and FI-ICR–MS (Fourier transform ion cyclotron resonance–MS), combined with mass spectrometry (MS) and NMR (nuclear magnetic resonance spectroscopy) [4–6]. Detection of metabolites is followed by multivariate statistical analysis of the accumulated datasets. Qualitative and quantitative changes in metabolic profiles

during development and under stress conditions are analyzed by metabolomics, a discipline known as a part of systems biology.

Aside from native metabolic alterations, metabolome profiling is used to detect the changes in plant metabolism brought about by genetic engineering. Such improvements in metabolites serve to elevate the economic importance and environmental sustainability of agricultural plants by increasing herbicide tolerance and resistance to pests and pathogens. Plants possess a high biochemical potential of synthesizing an enormous number of various natural substances: fatty acids, phenolics, terpenoids, alkaloids, glucosinolates and other biologically active compounds; many of which are of great value to pharmacology [7]. Advances in metabolomics have enabled the decoding of many metabolic networks and provide for the active genetic engineering of pathways. These offer the opportunity to manipulate the biosynthesis of valuable, biologically active substances of interest, and to create "design" biochemicals. They also can serve to considerably alter the secondary metabolism thus improving a plant's capacities to synthesize new substances or, on the contrary, reduce their toxicity by switching off such metabolic pathways [8–10]. This is a direct effect of genetic engineering on the metabolome. Nevertheless, activation of one key enzyme will not always result in facilitation of the whole metabolic pathway. Thus metabolic profiling is an important tool to evaluate results of such genetic modification. The technology of genetic engineering is widely used in modern agriculture. Frequently plants are genetically engineered to improve their productivity or tolerance to adverse environment. Among the modified crops are maize, cotton, soybean, canola, rice, tomato, potato, among others [2, 11–13]. Other promising approaches are the use of biotech plant systems as a platform for the production of various heterologous proteins for the pharmaceutical industry [7, 14, 15]. The major advantage of plants as bioreactors, aside from their autotrophic type of nutrition, is that the edible plant with the recombinant protein of interest can be eaten directly, skipping rather expensive procedure of purifying the target protein. The production of immunogenic fragments of recombinant antigens in a bioreactor plant is called "plant-based vaccine". Apart from vaccines, biotech plants are used to synthesize numerous antibodies, cytokines, hormones, and other proteins [7, 14–16].

Social, ethical, economic, and ecological norms demand that biotech crops be subjected to intense scrutiny [17]. A very important question is whether accumulation of a large amount of "foreign protein" could be considered a stress factor by the engineered plant and be accompanied by an accumulation of compounds that would pose an ancillary potential risk. A series of investigations were focused on the evaluation of differences in the metabolic profiles of transgenic plants in comparison with wild types (WT). The main conclusion is that metabolism after transgenesis suffered only insignificant alterations or about the same in comparison to that of WT plants [12]. Moreover, results indicate that environmental variations usually produce greater major differences in metabolome composition than genetic modifications. To uncover a possible problem, we used inbred lines of *Nicotiana tabacum* L. plants synthesizing bovine interferon-gamma to study the effects of genetic transformation on the plant metabolome. It must be noted that interferon-gamma is a powerful stimulator of the immune system against pathogens and the tumors of various origins and has no enzymatic activity. Therefore its possible effect on metabolism would be indirect.

2. Material and methods

2.1 Plant material

In this study two separate inbred lines of tobacco harboring a heterologous gene of bovine interferon-gamma *Bt-sIFNG* under the control of a constitutive 35S

CaMV promoter were used. These lines were created earlier by the *Agrobacterium*-mediated genetic transformation of *Nicotiana tabacum* L., cv. Trabzon [18]. The transformation generated six independent kanamycin-resistant transgenic plants (T_0), of which only two became the founders of homozygous inbred lines: InterB.6 and Inter311.2 (T_1). They demonstrated stable inheritance and expression of the transgene insertion and presence of a biologically active heterologous interferon-gamma protein in plant tissues [18]. T_4 generation of transgenic plants, InterB.6.13.8-1 and Inter311.2.7.2-1, and WT tobacco (as a control) was used for the study. The Inter311.2.7.2-1 plant and WT were grown *in vivo* in soil culture in a greenhouse at a temperature of 22–25 °C with a 16 h photoperiod. InterB.6.13.8-4 and WT were cultivated *in vitro* in a MS0 medium [19] supplemented with sucrose (20 g/L) in sterile conditions under the same temperature and light conditions.

2.2 RNA extraction

Total plant RNA was extracted from tobacco leaves with a Pure-ZOL™ reagent according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Samples were then treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) (5 U/sample). After that the RNA was precipitated with ethanol to remove residual DNase I. Purified RNA was dissolved in sterile water and stored at −80 °C until analysis.

2.3 Reverse transcription

Two μg of total RNA were taken for cDNA synthesis using MMLV RT kit (Evrogen, Moscow, Russia) in accordance with manufacturer's protocol. cDNA samples were precipitated with 0.1 M sodium acetate in ethanol, diluted with sterile deionized water, aliquoted and stored at −80 °C until analysis.

2.4 PCR analysis

The PCR reaction mixture included Taq-polymerase (Evrogen), a mixture of 2 μM dNTP, magnesium-containing Taq-buffer, cDNA template (100–200 ng), the forward and reverse primers sIFNG-1 (5'-AGGAGTATGGACATCATCAAGCA-3′) and sIFNG-2 (5'-AGTCGTCGACCGGAATTTGA-3′) for *Bt-sIFNG* (product size 105 b.p.), and EF-1 (5'-CAAGCGGTCATTCAAGTATGC-3′) and EF-2 (5'-TGTCCAGGACGATCAATCACA-3′) for tobacco *Nt-EF-1α* gene (product size 135 b.p.) [20]. The tobacco house-keeping gene was used as a control present in both WT and transgenic plants. The primers were ordered from Evrogen. Amplification of the fragments corresponding to *sIFNG* and *Nt-EF-1α* genes was performed using the following program:

95°C, 2 min —
{95°C, 20 s,
60°C, 30 s,
72°C, 30 s} — 32 cycles,
72°C, 1 min

in CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories).

2.5 Agarose gel electrophoresis

Electrophoresis was performed in a 1% agarose gel in a TAE buffer with the addition of ethidium bromide (0.5 μg/mL) for 40 min at a voltage of 120 V. PCR fragments were visualized in UV light.

2.6 Sample preparation for metabolome analysis

Average samples of 3–5 leaves were prepared in four biological replicates. The plant material (0.2 g) was frozen in microtubes with liquid nitrogen and ground three times in a Tissue Lyser LT (Quiagen, Düsseldorf, Germany) bead mill with metal balls 5 mm in diameter (50 hits/s, 2 min) and subjected to a single-stage extraction with two mL of methanol. After the centrifugation at 15,000 g for 15 min at 4 °C the supernatant was collected and evaporated in a refrigerated CentriVap centrifugal concentrator (Labconco, Kansas City, MO, USA) at 10 °C. The dried residue was dissolved in pyridine with tricosane (nC23) as an internal standard. The samples were then supplied with the silylating agent BSTFA: TMCS 99:1 (Sigma-Aldrich, St. Louis, MO, USA) and derivatizated at 90 °C for 20 min [21, 22].

2.7 Gas chromatography and mass spectrometry (GC–MS)

An Agilent 5860 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with Agilent 7893 autosampler, and Agilent ChemStation E.02.02.1431 software were used for analysis. The samples were separated on a J&W HP-5MS capillary column 30 m long, 0.25 mm in diameter, stationary phase film (95% dimethylpolyoxane, 5% diphenyl), thickness 0.1 mm. The helium gas constant flow was 1 mL/min and the inlet temperature was 250 °C. The temperature parameters of the oven included an initial temperature of 70 °C and a linear increase to 320 °C at the rate of 4 °C/min. Chromatograms were registered with an Agilent 5975C mass selective detector. Metabolite profiling was performed using equipment of the Center for Molecular and Cell Technologies of Research park of St. Petersburg State University.

2.8 GC/MS data processing

GC–MS data was processed using the PARADISe program (Department of Food Science Faculty of Science, University of Copenhagen, Denmark, [23]) coupled with NIST MS Search (National Institute of Standards and Technology (NIST), USA). In addition, the AMDIS (Automated Mass Spectral Deconvolution and Identification System, NIST, USA) was used. The following mass-spectrometer libraries were applied: NIST2010, library of the Resource Center of the Science Park "Center for Molecular and Cell Technologies" (St. Petersburg State University), the Golm Metabolome Database (GMD) and MoNA (Massbank of North America). Retention index (RI) was determined by calibration with standard alkanes.

2.9 Data analysis and visualization

Data analysis was performed in the environment of the R language 3.6.3 "Holding the Windsock" [24]. Data were normalized by internal standard (nC23) as well as by sample median. The data were log-transformed and standardized. Metabolite that was not detected but present in other replicated samples was considered a technical error and missing values were imputed. Missing data imputation was performed by KNN (k-nearest neighbors) with an *impute* R package [25]. The heatmap was made with ComplexHeatmap [26]. PCA (Principal Component Analysis) was realized with *pcaMethods* [27]. OPLS-DA was performed in the *ropls* package [28].

For enrichment analysis, the *fgsea* package was used [29]. For the statistical ranking factor, loadings of the predictive components from OPLS-DA models were used. Pathways associated with this metabolite set were extracted from KEGG with the *KEGGREST* package using *Nicotiana tabacum* as a reference organism [30]. The resulting lists of metabolites for pathways were manually curated. Metabolites identified

just up to class were included in the pathways related to these groups. Pathways were mapped within the Cytoscape software environment [31]. Graph nodes were assigned to KEGG pathways, edges represent the presence of common metabolites.

3. Results and discussion

Previous studies generated two different families of transgenic tobacco harboring the heterologous gene of bovine interferon-gamma *Bt-sIFNG* [18, 32]. One homozygous *Bt-sIFNG* plant was identified in the Inter311.2 family at T_1 generation. It established the founder of the 311.2 inbred line of transgenic *Bt-sIFNG* tobacco. The other was obtained in T_2 generation of the InterB.6.13 family, leading to a second inbred line of tobacco synthesizing interferon-gamma. In the current study, plants from the T_4 generation of transgenic lines, Inter311.2.7.2-1 and InterB.6.13.8-1, as well as wild type (WT) tobacco for control were used. To demonstrate the transgenic nature of 311.2.7 and B.6.13, RNA was extracted and reverse transcribed. RT-PCR was performed with primers for the tobacco gene *Nt-EF-1α*, encoding housekeeping protein elongation factor 1α, and for the *Bt-sIFNG* gene, encoding bovine interferon-gamma. It was clear that PCR products corresponding to *Nt-EF-1α* were observed for cDNAs from all tested plants, both WT and transgenic. RT-PCR products with primers for *Bt-sIFNG* gene were found in transgenic plants only (**Figure 1**).

Therefore plants of transgenic inbred lines used in the study were shown to possess and express *Bt-sIFNG* gene insertion. These plants synthesize heterologous interferon-gamma protein at about 1 to 1.5 μg per g of fresh weight of plant tissue [18]. The biological effects of plant-produced bovine interferon-gamma demonstrated antiviral activity in bovine cell culture and the induction of immune response in mice, manifested in qualitative alteration of peripheral blood lymphocytes and overall level of Ig G [18].

The production of recombinant human interferon-gamma expressed in *Escherichia coli* resulted in accumulation of 0.35 g/g of dry weight [33]. Similarly *E. coli* culture produced human interferon-gamma with a speed 3 mg/L in hour [34]. Apart from bacteria, different yeast systems based on transformed lines of *Pichia pastoris* and *Saccharomyces cerevisiae* are used for interferon production of up to 300 mg/L [34]. Several examples using insect or even mammalian cell lines have also been designed. Thus in spite of all the economic costs for production and further purification, all of are characterized by rather intensive protein synthesis.

Another interferon-producing system on the basis of rice suspension culture was shown to accumulate intracellular human interferon-gamma protein of up to

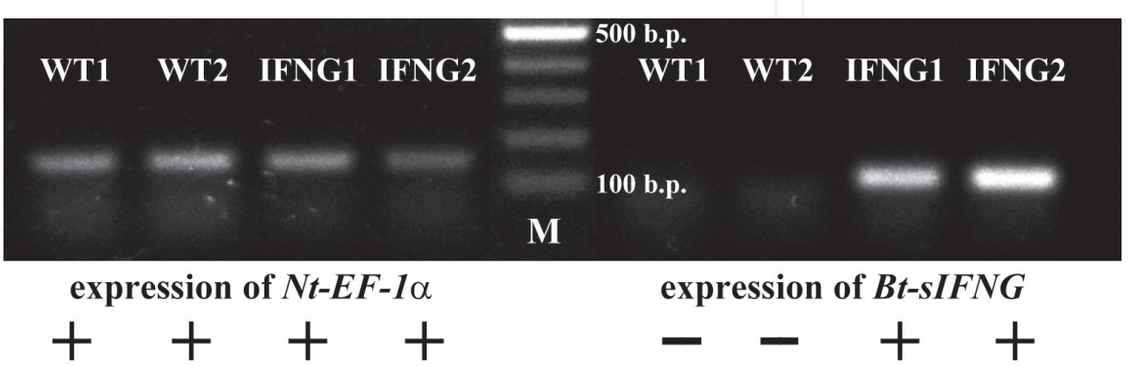

Figure 1.
Electrophoresis of RT-PCR products of cDNAs obtained from WT plant grown in a pot (WT1), or in vitro (WT2), transgenic 311.2.7.2-1 plant, grown in a pot (IFNG1), and transgenic B6.13.8-1 plant, cultivated in vitro (IFNG2), with primers for tobacco gene Nt-EF-1α, or heterologous gene Bt-sIFNG. M - DNA ladder (100–500 b.p.).

699.79 ng/g of cells [35]. The most effective genetic construction used was starvation-inducible endogenous rice αAmy3 promoter.

An example of intensive synthesis of functionally active endogenous plant protein in leaf cells is the accumulation of the major photosynthetic enzyme – RUBISCO (D-Ribulose-1,5-bisphosphate carboxylase/oxygenase), which is claimed to be the most abundant plant protein on earth, even if recently the exact value of the carboxylase has come under revision [36]. The amount of the RUBISCO protein ranged from 30 to 50% of total soluble protein in leaf cell.

Synthesis of protein due to heterologous expression might seriously affect amino acid balance and/or cause alterations in carbohydrates as sources of energy. But along with that, an unregulated accumulation of "foreign" protein inside a plant cell could become a stress signal itself which would lead to production of defensive metabolites. For example, in microbial-based bioreactor systems overproduction of recombinant protein led to formation of an insoluble protein bodies in cytosol and development of oxidative stress as was shown in *E. coli* [37], *P. pastoris* and *S. cerevisiae* [38]. Thus it is appropriate to assume that the tobacco plant metabolism might be shifted due to transgenesis. Earlier comprehensive metabolic analysis of transformed plants showed different alterations in various biochemical processes, nevertheless there was not a clearly established link between gene manipulation and changes in metabolism [12]. The aim of this work was to distinguish possible rearrangements of tobacco leaf cells' metabolic profiles due to transformation with a heterologous gene of bovine interferon-gamma *Bt-sIFNG* under the control of a constitutive 35S CaMV promoter. It was decided that additional attention should be focused on two types of cultivation (in pot soil culture and *in vitro* sterile conditions) – both suitable for transgenic plants growing to estimates of their effect on metabolic alterations.

Metabolite profiles of tobacco leaves were performed by GS-MS analysis. In total profiles included 350 metabolites (**Figure 2**). 80 of these were identified up to the exact metabolite and 150 were identified up to the metabolite class. Metabolic profiles were characterized by a wide variety of carbohydrates and their derivatives (about 90), including pentoses, hexoses and oligosaccharides and their derivatives such as sugar alcohols and sugar acids. This pattern is typical for profiling plant metabolites. The profiles were completed with 19 amino acids, including proteinogenic ones; about 20 carboxylic acids, mainly energy metabolism intermediates; and quite a few fatty acids and their derivatives (only 8). Moreover, some secondary metabolites were also identified.

Simple unsupervised dimension reduction methods (PCA, **Figure 3**) showed differences in tobacco leaf metabolome due to both the type of growth and to genetic status. Metabolite profiles are visualized in the score space of the first two principal components (PC). Metabolomes were grouped along to PC1 according to the type of growth (35% dispersion) and were grouped along with PC2 accordingly to the absence/presence of *Bt-sIFNG* gene insertion (28% dispersion). Similar metabolite profile grouping was also characterized by a correlation of metabolite content. Spearman's correlation coefficient was used as a measure of distance (1-r) and multidimensional scaling (MDS) to reduce the dimension (**Figure 4**). The obtained results highlighted the importance of both factors, namely the method of plant cultivation and its transgenic status.

Further comparison of metabolite profiles of WT and Inter311.2.7.2-1 transgenic tobacco plants grown in pots was detailed by supervised methods such as PLS-DA. The predictive component of PLS-DA has a 31% rate of dispersion (R2Y = 0.99, Q2Y = 0.83). So the metabolic shifts prompted by the transgenic construct were significant. A bar plot of factor loadings of the predictive component from OPLS-DA is presented in **Figure 5**. Positive values correspond to higher content in the WT. According to results, leaves of the WT tobacco plant contain a higher

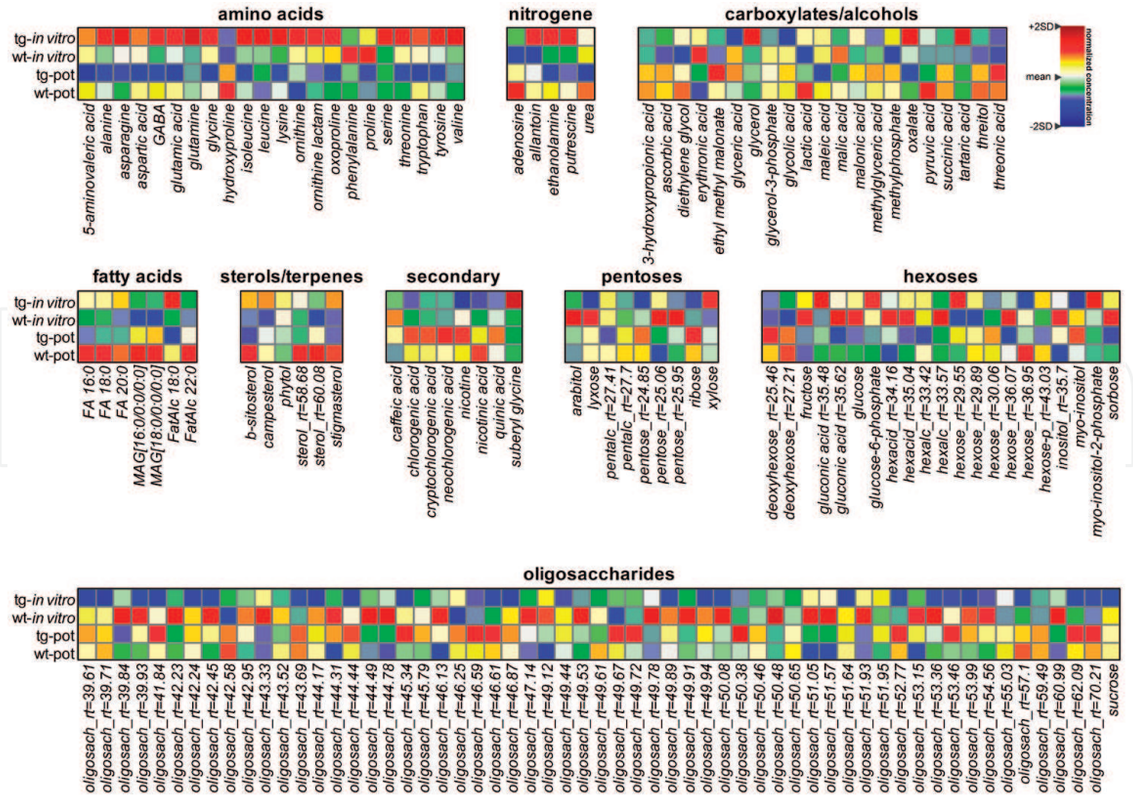

Figure 2.
Heatmap of metabolite content in WT and transgenic (tg) tobacco plants grown in pot or in vitro culture (logarithmic, normalized to median and standardized values).

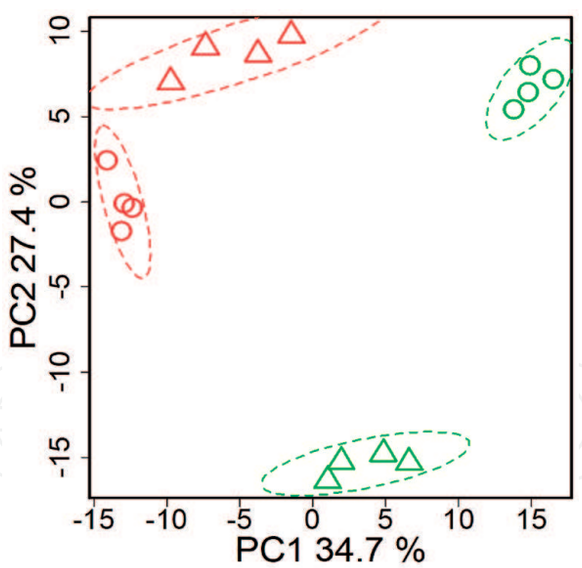

Figure 3.
Representation of metabolite profiles of WT (Δ) and transgenic (o) tobacco in low-dimensional spaces. PCA score plot, % - percent of variance, ellipses - 90% CI. red - pot cultivation, green - in vitro cultivation.

amount of lipophilic compounds, including acylglycerols, sterols, and some fatty acids. WT was also characterized by a higher content of amino acids, amines and carboxylates. Leaves of transgenic plants accumulated high levels of oligosaccharides. Subsequent enrichment analysis revealed metabolic processes that occurred in WT and transgenic tobacco plants (**Figure 6**). In agreement with the bar plot, the results of enrichment analysis of WT plants indicated an intensive wide spectrum of metabolic pathways responsive for the balance of amino acids, carboxylates, lipophilic metabolites, and others. Furthermore, biotech tobacco was characterized

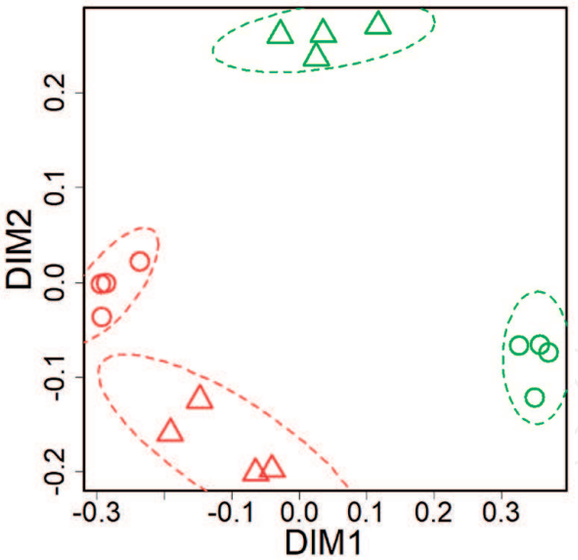

Figure 4.
Representation of metabolite profiles in low-dimensional spaces. Metabolite profiles in the space revealed using MDS with 1-rho as a measure of distance, where rho is Spearman's correlation coefficient, ellipses - 90% CI. Δ - wt, o - transgenic, red - pot culture, green - in vitro cultivation.

by an intensification of only the carbohydrate metabolism pathways. Thus the procedure of growing a plant in soil pot culture exerted significant effects on its metabolism. Such cultivation activated different aspects of WT plant metabolism involving the developmental activity of young leaves. Surprisingly, the presence of an interferon-gamma synthesis-encoding heterologous construct intensified carbohydrate metabolism. The possible higher amount of amino acids required for excessive synthesis of heterologous protein were not distinguished, perhaps because of the intensification of protein synthesis itself.

Another pair of plants were grown in sterile conditions with the application of *in vitro* technology that provides a full supply of required nutrients. Therefore the next step was a similar comparative analysis of metabolite profiles of WT and InterB.6.13.8-1 transgenic plants cultivated *in vitro*. The OPLS-DA provided showed that 65% of the dispersion was associated with the predictive component (R2Y = 1.0, Q2Y = 0.98). Thus the influence of the genotype is more pronounced under *in vitro* conditions. The bar plot of factor loadings of the predictive component from OPLS-DA is presented in **Figures 2** and **7**. Leaves of the WT plant accumulated different sugars, oligosaccharides, a number of amino acids, and lipophilic compounds. The transgenic plant, in contrast to pot cultivation, was characterized by a higher diversity of metabolite classes. Metabolic profiles consist of a large number of amino acids, carboxylates, sterols (stigmasterol, β-sitosterol and campesterol), as well as other lipophilic compounds. Nonetheless, enrichment analysis proved activation of only those pathways related to amino acids in transgenic plants and pathways of sugar metabolism in WT plants (**Figure 8**).

Taken together data obtained for WT and transgenic plants grown in pots and *in vitro* were used for SUS-plot construction (**Figure 9**). Unexpectedly, the effect of transformation was the opposite at different conditions of plant cultivation. It might be assumed that the rich conditions of *in vitro* growth cause more intensive metabolic alterations due to genetic modification. These more "comfortable" conditions for plant development might enhance the intensification of heterologous gene expression and interferon-gamma synthesis. This supposition is indirectly confirmed by higher accumulation of PCR-products of the *Bt-sIFNG* transcript in the leaves of transgenic tobacco grown *in vitro* (**Figure 1**).

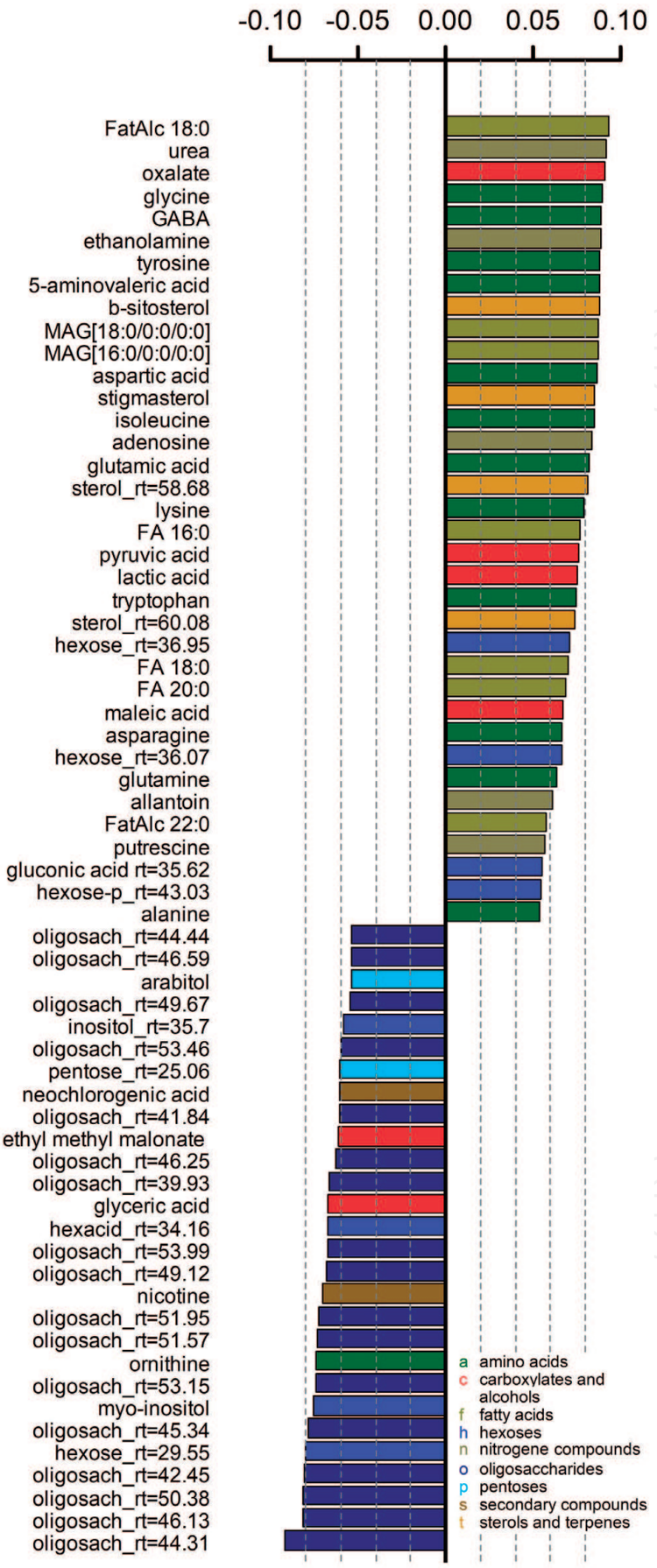

Figure 5.
Differences between metabolomes of WT and transgenic plants cultivated in pots. Barplot of factor loadings of the predictive component from OPLS-DA. Positive values correspond to a higher content in the wild type. Colors mirror chemical class (legend same as in **Figures 7** *and* **9**). *Abbreviations: rt. - retention time, oligosacch -oligosaccharides, FA - fatty acid, FatAlc - fatty alcohols, hexacid, pentacid, hexalc, pentalc - sugar acids and alcohols, MAG - monoacylglycerols.*

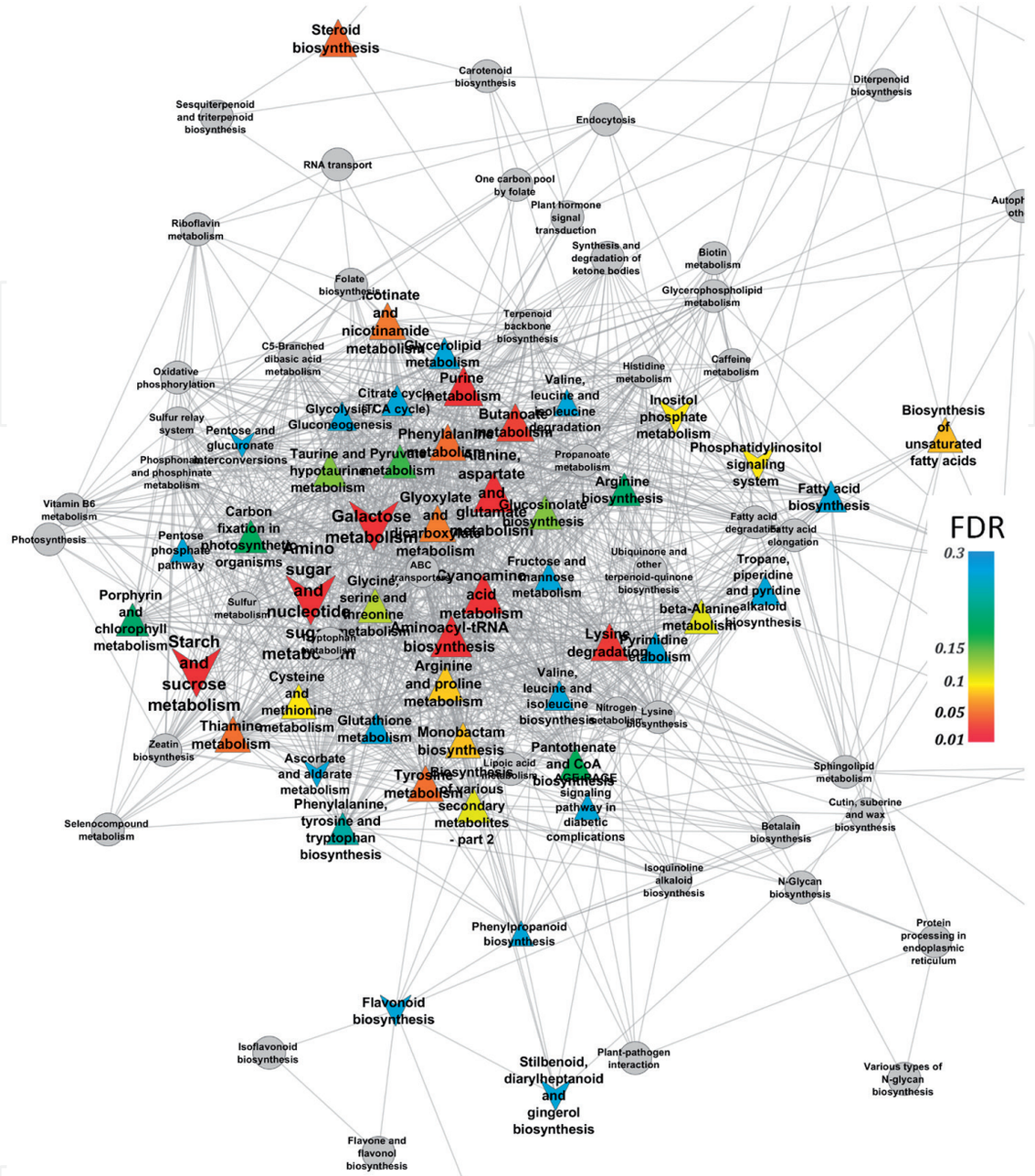

Figure 6.
Enrichment analysis based on predictive component loadings from OPLS-DA model for WT and transgenic tobacco plants cultivated in pots. The network of biochemical pathways (nodes) of tobacco, if the paths have common metabolites, then they are connected by edges. Colors mark significance of enrichment (FDR - false discovery rate), size reflects level of enrichment (|NES| - normalized enrichment score), upward direction - activation (NES > 0) in WT, downward - repression (NES <0) in WT.

An additional criterion that would be of an interest is the crosslink between different metabolic pathways. Thus one can expect some correlative alterations in metabolite content. Changes in the correlations of the metabolite content may reflect systemic metabolic changes. Therefore, we examined the frequency distribution of the correlation coefficients in plants of different genetic status, grown under different conditions. Based on the analysis of the frequency distribution of the Pearson correlation coefficient (**Figure 10**), it was determined that in transgenic plants the number of strong correlations increases when cultivated in a pot in comparison with *in vitro* conditions. The observed effect was absent in WT plants. This might reflect the more stressful cultivation conditions for transgenic plants in pot culture. It may be assumed that the increase in correlations is associated with some kind of coordinated response to stress.

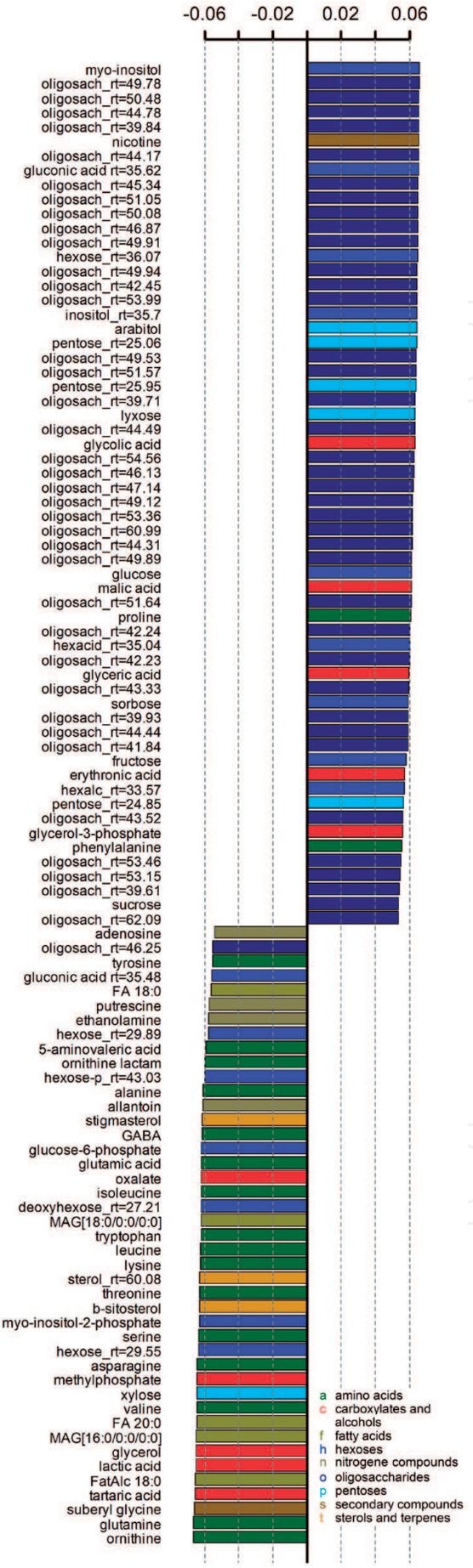

Figure 7.
Differences between metabolomes of WT and transgenic plants cultivated in vitro. Barplot of factor loadings of the predictive component from OPLS-DA. Positive values correspond to a higher content in the wild type. Colors mirror chemical class. Legend and abbreviations as in **Figure 5**.

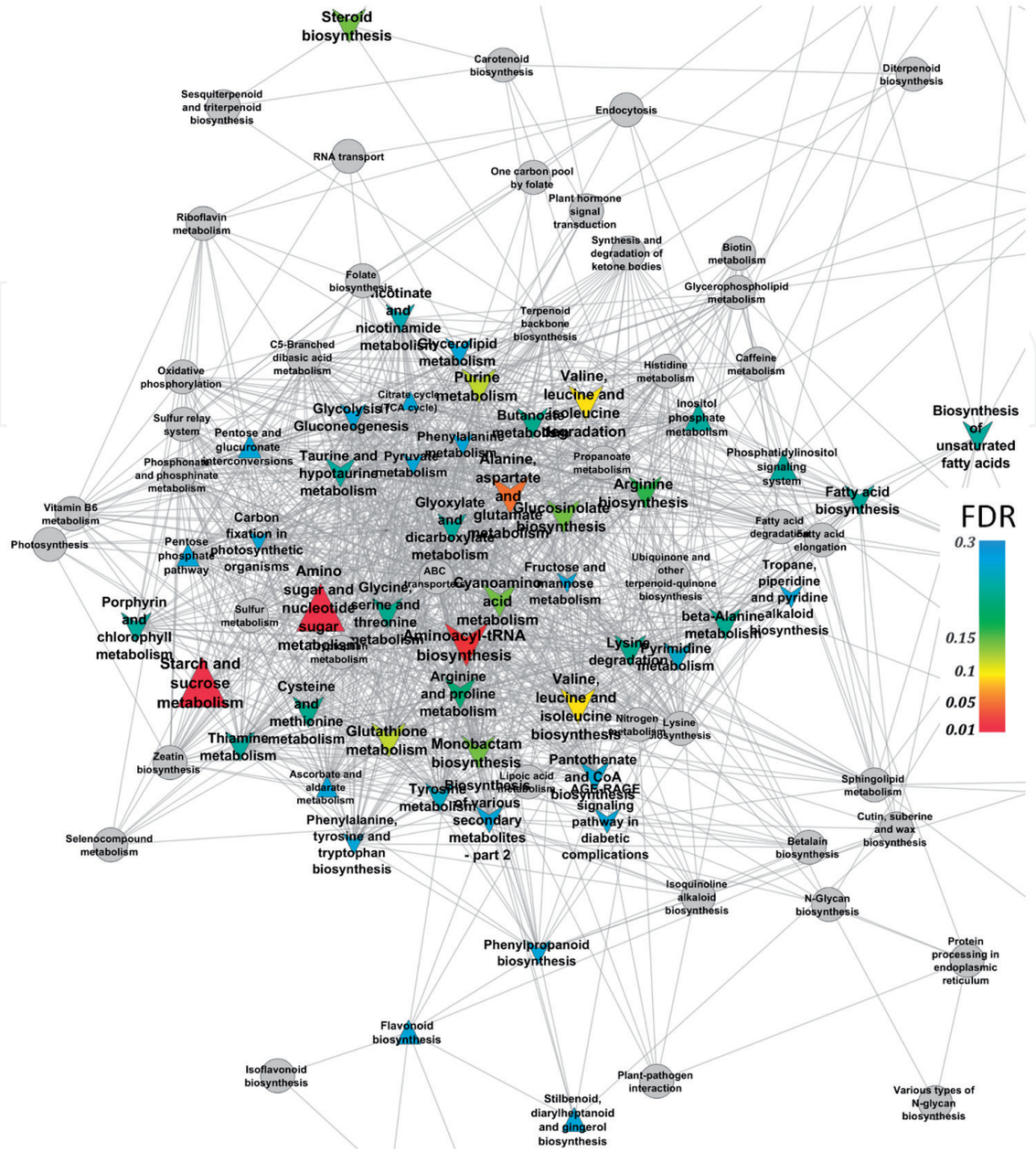

Figure 8.
Enrichment analysis based on predictive component loadings from OPLS-DA model for WT and transgenic plants cultivated in vitro. The network of biochemical pathways (nodes) of tobacco, if the paths have common metabolites, then they are connected by edges. Colors mark significance of enrichment (FDR), size reflects level of enrichment (|NES|), upward direction - activation (NES > 0) in WT, downward - repression (NES <0) in WT.

Analysis of selected literature sources revealed that the majority of work on genetically-modified plant metabolome was carried out with plants expressing heterologous genes encoding different enzymes [12]; i.e. these types of transgenes directly affect the metabolome by enhancing pathways operated by the target enzyme protein. The bovine interferon-gamma used in this study belongs to cytokines and has no enzymatic activity. Therefore, its effects on metabolism would be indirect. There is no doubt that introduction of excessively synthesized "foreign" protein would interfere with the cellular metabolism at least at the level of consumption of an additional amount of amino acids and energy (i.e., sugars and carboxylates). The results obtained showed accumulation of oligosaccharides in the transgenic plant Inter311.2.7.2-1 (compared to WT), grown in soil pot culture (**Figures 2** and **5**) and stimulation of amino acid and sterol metabolism in InterB.6.13.8-1 cultivated *in vitro* in Murashige-Skoog medium (**Figures 2** and 7).

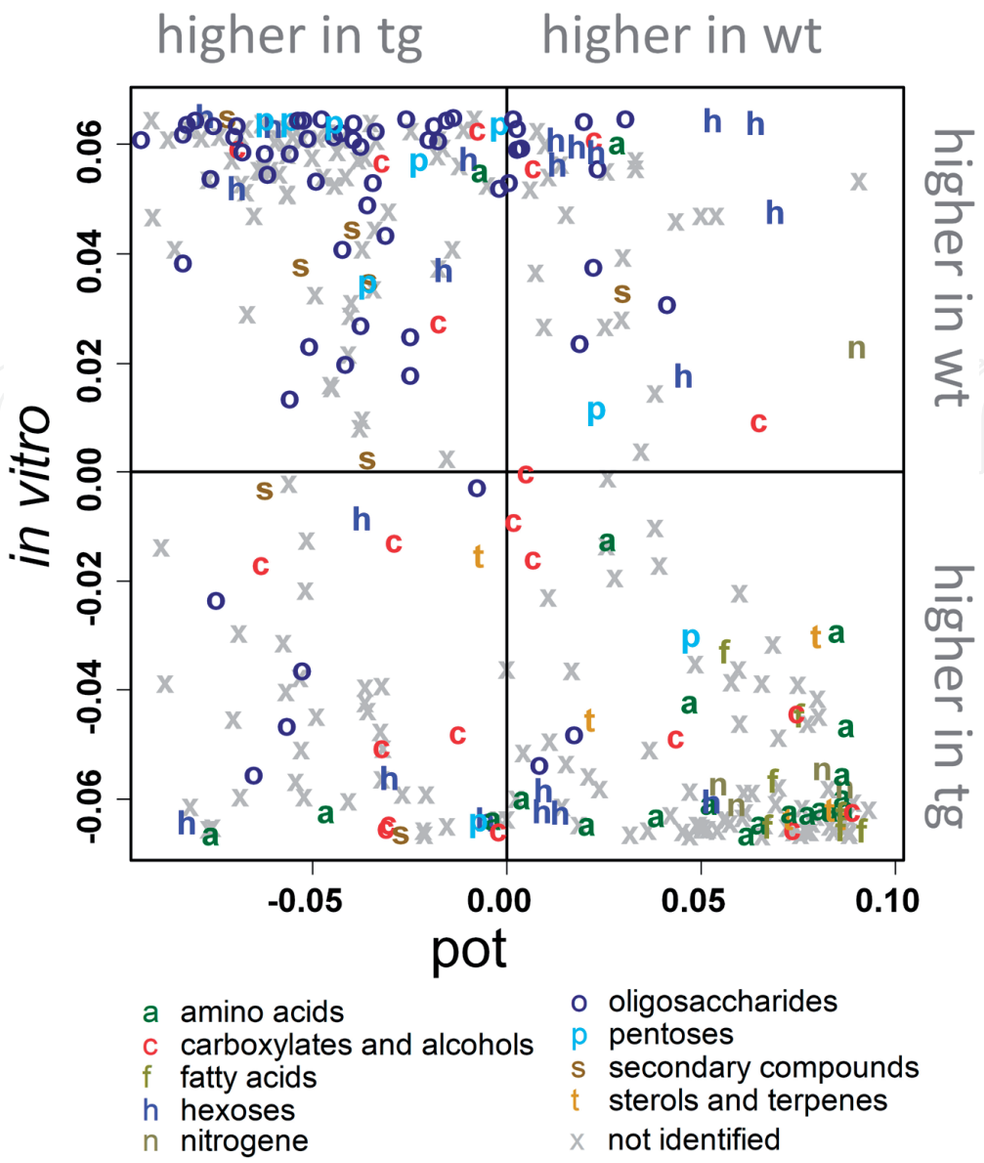

Figure 9.
Comparative analysis of the transformation effects in vitro and in the pot culture. SUS-plot of predictive loadings of OPLS-DA models for WT and transgenic (tg) plants comparison during pot and in vitro cultivation. Colored letters represent chemical classes.

We did not succeed in finding any report on effects of heterologous interferon-gamma on the metabolome of its bioreactor organism, neither of plant, nor of animal origin. Exogenously applied human interferon-gamma led to accumulation of some amino acids (Ala, Tyr, etc.) and depletion of pyruvate and lactate in the culture of human mesenchymal stem cells [39]. Despite the fact that tobacco is a non-target organism for bovine interferon-gamma, its synthesis in InterB.6.13.8–1 grown *in vitro* also led to significant accumulation of amino acids (Lys, GABA, Trp, Asp, Asn, Ser, Glu, Gln, Gly, Tyr, Ile, Leu, Val, Ala, Thr and oxoproline); levels of lactic and pyruvic acids were more or less the same in transgenic plants as in those compared with WT (**Figures 2** and 7).

There are quite a few studies of the metabolome of transgenic plants synthesizing non-enzymatic heterologous protein which can be compared with the system producing bovine interferon-gamma and they mostly concern different types of Bt-toxin expressing plants [12, 13, 40]. It was shown that both cultivation conditions and gene modification induced similar alterations of metabolomes. Moreover, in many cases the growing conditions or developmental stage of plants had a greater effect on the metabolome than the presence of a transgenic insert.

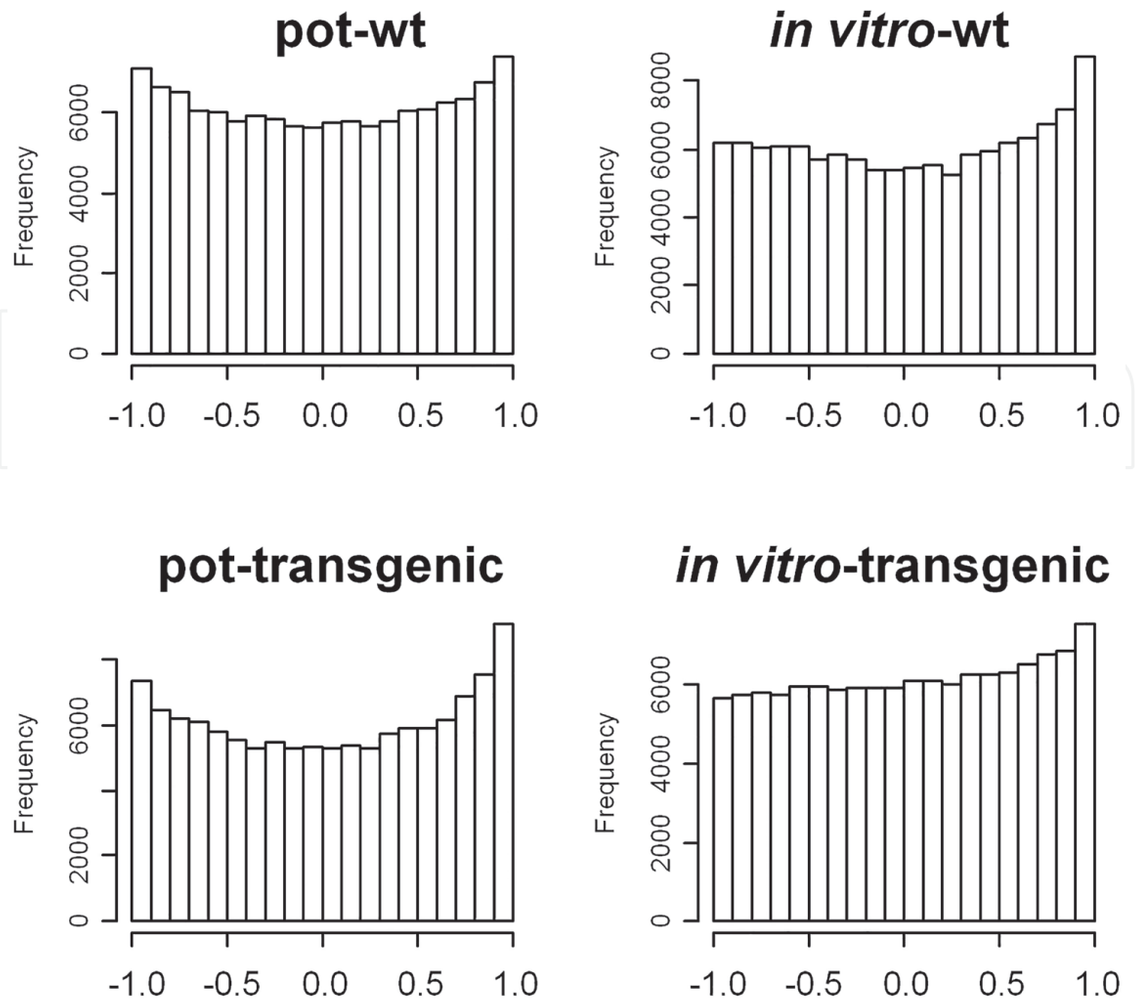

Figure 10.
Influence of transformation and cultivation method on the correlation of metabolite content. Histograms of frequencies of Pearson's correlation coefficient.

So further investigations of biotech plant metabolomics are strongly required, especially those focused on the metabolomics of bioreactor plants.

4. Conclusion

Taken together, the data of this investigation clearly showed that metabolic profiles are dynamic parameters which characterize plant development. Metabolic profile changes in tobacco plants specialized in the synthesis of bovine interferon-gamma were discovered. The presence of heterologous of *Bt-sIFNG* gene insertion itself did not cause development of intracellular stress in transgenic tobacco. In fact, even more intensive metabolic alterations were determined to be dependent on the type of plant growing conditions. It was assumed that soil conditions might be considered as somewhat stressful for transgenic bioreactor plants in comparison with cultivation *in vitro*. This could cause depletion of the target gene transcript accumulation and thus indirectly indicate active regulation of "foreign" protein synthesis by the host transgenic plant. The results obtained are in accordance with data in the literature illustrating the priority of growing conditions and developmental stage above transgenic status in the determination of metabolic intensity. Nevertheless, it is too early in the resolution of this question to make firm conclusions and further investigation is still needed.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviation

FDR	false discovery rate
GC–MS	gas chromatography–mass spectrometry
MDS	multidimensional scaling
NES	normalized enrichment score
OPLS-DA	orthogonal partial least squares discriminant analysis
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PLS-DA	partial least squares discriminant analysis
RI	retention index
SUS	shared and unique structures
T_X generation	generation of transgenic plants
T_0	transformed plants
T_1	first generation, obtained after self-pollination of T_0 plants
T_2	second generation, obtained after self-pollination of T_1 plants, etc.
WT	wild type

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