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Genetic enhancement of carbohydrate and lipid metabolism through overexpression of Arabidopsis thaliana *diacylglycerol acyltransferase 1 (AtDGAT1)* gene in transgenic Jatropha (*Jatropha curcas*)

Devendra Kumar Maravi

devendrakumaravi@gmail.com

Shaheed Mahendra Karma University Bastar

Vaibhav V Goud

Indian Institute of Technology Guwahati

Nozomu Sakurai

Kazusa DNA Research Institute

Ajaya Kumar Biswal

University of Georgia

Lingaraj Sahoo

Indian Institute of Technology Guwahati

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Abstract

Jatropha curcas is a potential biofuel crop due to the presence of high amount of oil in its seeds making it a suitable biodiesel feedstock. Triacylglycerol (TAG) is the most abundant energy reservoir in Jatropha. Insertion of transgene can cause unpredicted effects by altering the expression of endogenous genes triggering metabolic changes and ultimately plants phenotype. The metabolomics approach is countable to evaluate Jatropha following genetic manipulation. The fundamental aim was to carry out metabolite profiling of transgenic Jatropha expressing *Arabidopsis thaliana DGAT1* gene (*AtDGAT1*), encoding an enzyme which catalyses the final and only committed step in TAG biosynthesis. The NMR-based metabolomics approach was employed to identify the altered metabolites after the genetic modification. Metaboanalyst 5.0 was used to statistically analyse the data and determine the changes in metabolic pathways in the transgenic and wild-type Jatropha. The results revealed that the *AtDGAT1* gene induced intended and unintended metabolic variations in the carbohydrate and lipid metabolism in the transgenic lines. ^{1}H NMR-based metabolomics revealed the metabolite variations among the *AtDGAT1* expressing transgenic lines and identified the altered metabolic pathways.

Introduction

Jatropha curcas L (Jatropha) is a tropical and subtropical shrub or tree from the *Euphorbiaceae* family. Jatropha is a non-edible oilseed crop, holding great interest for biodiesel production. The seeds have 30–45% oil content by weight, in the form of triacylglycerides (TAGs), which is composed of three fatty acids esterified to the glycerol backbone. The fatty acids are mainly unsaturated consisting of oleic acid (C18:1, ~ 40.1%) and linoleic acid (C18:2, ~ 37.6%), as well as saturated, like palmitic acid (C16:0), stearic acid (C18:0), and others [1]. TAGs are an important part of the bio-economy by providing a source of highly reduced carbon for both food and non-food applications, and simultaneously supplying feedstock to produce petrochemical alternatives [2, 3]. Jatropha oil can be used directly after extraction in diesel generators and engines or can be refined to produce fatty acid methyl esters (FAMEs) by the transesterification process. Apart from seeds, other tissue types like leaves, roots, and stems have also has been extensively studied for various industrial and medicinal applications [4, 5].

Lipid metabolism is closely connected to carbohydrate metabolism [6]. They can be hydrolyzed to produce glycerol and fatty acids. Since glycerol is three-carbon alcohol, it is metabolized quite readily into an intermediate in glycolysis called dihydroxyacetone phosphate (DHAP). The dihydroxyacetone, obtained from glycerol can be converted into pyruvic acid through the glycolysis pathway to generate energy [7, 8]. In addition, dihydroxyacetone may also be used in gluconeogenesis to make glucose-6-phosphate for glucose. Fatty acids are oxidized to acetyl-CoA which is converted into ATP, CO_2, and H_2O using the citric acid (TCA) cycle and electron transport chain [9]. Fatty acids and lipids are synthesized from carbohydrates and occasionally from proteins. Depending upon the energy requirements, the acetyl-CoA enters the TCA cycle or is used to synthesize fatty acids. Triacylglyceride biosynthesis is a part of glycerolipid biosynthesis [10, 11, 12]. The only committed and rate-limiting step in this process is the acylation of the diacylglyceride using acyl-CoA substrates, catalyzed by a diacylglycerol

acyltransferase enzyme, which is encoded by *diacylglycerol O-acyltransferase gene* (*DGAT*) [13, 14, 15]. In Arabidopsis, the *AtDGAT1* gene is essential for triacylglycerol synthesis in seeds and the normal development of both seeds and pollen. *AtDGAT1* deficiency also alters carbohydrate metabolism [16, 17].

The extensive ranges of the biochemical processes that occur inside the cells suggest a need for a correlated investigation of interdependent biochemical pathways providing an overview of the entire metabolism within an organism [18]. Currently, the study of metabolomes representing the intermediates and end-products of various cellular processes is of great interest. Unlike the genome-wide study at the transcription and translational level, metabolomics study interprets the physiology of a cell, tissue, or organ at an instant. In the recent past, a great determination has been applied to the metabolomics approach to investigate the compositional equivalence between genetically modified and unmodified crops. The metabolomics study, under various developmental conditions, has been reported in Jatropha by many groups [19, 20, 21]. Due to the complexity and diversity of plant metabolites, it is not possible to get complete information about the whole plant metabolites using a single analytical method. Spectroscopy methods such as proton nuclear magnetic resonance (^{1}H NMR), a powerful tool to analyze and quantify the metabolites in solution or cell extracts. It is a non-destructive technique requiring a minimal amount of sample preparation, with no need f for derivatization. However, when high-resolution NMR techniques are coupled with multivariate statistical analysis, resulting partitions give unambiguous information. Several ^{1}H NMR studies have been conducted on the analysis of plant metabolites [22, 23, 24, 25, 26] and genetically modified plant metabolomics [27, 28, 29, 30]. However, little information is available for the metabolomics study of genetically engineered Jatropha cultivars, a feedstock for the production of bio-diesel.

In a previous study, we have reported that transgenic Jatropha ectopically expressing *Arabidopsis DGAT1* gene showed a dramatic increase in lipid content by 1.5- 2.0fold in leaves and 20–30% in seeds, and an overall increase in TAG and lower free fatty acid (FFA) levels compared to the wild-type plants [31]. However, the insertion of a transgene to increase the accumulation of TAGs may have various unpredicted effects on the expression of endogenous genes that lead to triggering changes in plant metabolomic pathways. To explore that, we conducted a metabolomics analysis of transgenic Jatropha constitutively overexpressing the *AtDGAT1* gene and its non-transgenic counterpart. In this study, we performed non-targeted metabolite profiling of Jatropha leaves by ^{1}H-NMR with the combination of multivariate analysis to identify changes in the leaf metabolome following the genetic manipulation and predicted its impact on metabolic and energy pathways.

Material and Methods

Plant materials and metabolite extraction

Wild type Jatropha was genetically modified by introducing *Arabidopsis thaliana* diacylglycerol O-acyltransferase1 gene (*AtDGAT1*) via *Agrobacterium*-mediated transformation, using a hyper-virulent *Agrobacterium* strain, EHA105 harbouring pBI121 35S::*AtDGAT1* construct. The detailed procedure for

genetic modification has been extensively described previously [31]. Young leaves were harvested and immediately frozen in liquid nitrogen. The leaves were ground to powder, followed by freeze-drying in lyophilizer (Thermo Fisher Scientific, Waltham, USA), and stored in -80^{o} C until further analysis.

For metabolite extraction, the powdered leaf sample was dissolved in methanol (MeOH, Merck) in a 1:5 (w/v, solvent ratio mg/µl) ratio by vortexing followed by sonication for 15 min at room temperature. Any precipitate was removed by centrifugation at 12000 rpm for 10 min at 4^{o}C, and the supernatant was carefully collected and dried in SpeedVac concentrator (Eppendorf AG, Hamburg, Germany) for 2 hrs. The dried extract was then dissolved in deuterated chloroform ($CDCl_3$, D:99.8%, Merck) containing 0.01% tri-methylsilyl propionic acid-d4 sodium salt (TSP-d4, Merck) serving as internal standard. 600 µl of the resulting solution was loaded into a 5mm NMR tube for analysis. The extraction procedure was repeated thrice from the same set of freeze-dried samples for reproducibility.

^{1} H-NMR spectroscopic analysis

The ^{1}H-NMR experiment was performed on Bruker Avance III 600 MHz spectrometer (BrukerBiospin GmbH, Reinsteten, Germany), operating at 600.17 MHz at 300 K temperature, with 900 pulse widths at 5.75 sec, relaxation delay of 1 sec, 16 scans, 64 K data point, and a spectral width of 17023 Hz. The raw data was acquired in the form of free induction decay (FID) using Topspin 2.1 software for Bruker Avance systems. All the data were obtained using a spectral width of 16.0 ppm with an acquisition time of 3sec.

Data processing and multivariate analysis

The FIDs were imported to the processor of Chenomx NMR suite 8.2 (http://www.chenomx.com/). The spectrum was automatically Fourier transformed, with an exponential line broadening factor of 0.3 Hz followed by automatic phasing and polynomial baseline correction. The resulting spectrum was calibrated to the standard peak (TSP-d4 at δ = 0.00 ppm) to give a desired spectral region (0.00 ppm to 10.00 ppm). To clean the data, the peaks for residual water and solvent were removed (water, 1.56 ppm; methanol, 3.48 ppm; and $CDCl_3$, 7.24–7.26 ppm). The processed spectrum was imported to the profiler of Chenomx NMR suite 8.2 (http://www.chenomx.com/) for metabolite identification and quantification. First, binning was performed with a spectral width of 0.04 ppm (12 Hz for 600 Hz NMR). To ensure that all the spectra correspond to the same overall concentration, the binned dataset of each spectrum was normalized by the total area (%) method which records bin values as a fraction of the total spectrum area [23, 32]. Using the Chenomx reference library representing spectral signatures of targeted compounds, along with the aid of published literature, metabolites and functional groups were predicted by assigning the chemical shift limits or values [33]. The concentration of the metabolites was estimated by calculating the peak area using the assigned chemical shift limits as the integral limits.

To study any variation in the profile of the identified target metabolites, multivariate data analysis was used. The concentration values of the triplicate experiments were imported to MetaboAnalyst 4.0

software [34], and the data were log-transformed, mean-centered, and auto-scaled. Principal component analysis (PCA) and partial least square-discrimination analysis was performed to give an intrinsic overview of the data set and reveal possible groups and outliers. Orthogonal partial least square-discriminate analysis (OPLS-DA) was used to evaluate with R^2X and Q^2 values representing the explained variations and model predictability respectively. Multiple algorithms were used to test and select statistically accurate and significant metabolites, like variable importance in projection (VIP) scores in the PLS-DA model, student's *t*-tests, and one-way analysis of variance (ANOVA). The heatmaps were generated using the 'correlation' and 'heatmap' modules.

Results and Discussion

^{1}H NMR spectra and metabolite identification

To investigate the effect of genetic modification on the metabolome of five transgenic Jatropha lines overexpressing *AtDGAT1*, ^{1}H-NMR was employed on the methanol extract of transgenic and wild type leaves. The variations in signal intensities in the NMR spectrum of the transgenic lines as that of wild-type Jatropha were observed. Chenomx database and previously published data were used to identify the compound in the leaf extracts. We identified 31 metabolites and functional groups representing carbohydrates, fatty acids, lipids, and lipid-precursors in the metabolic profile of the Jatropha leaves, playing a crucial role in energy metabolism and oil production (Table 1). The representative ^{1}H-NMR spectral regions in **Supplementary Fig. 1**, depict the signature peaks assigned to each metabolite or functional group. Their chemical shift values are listed in Table 1. The fatty acids were identified in regions of 0.9 ppm to 1.31 ppm. The lipid signals ranged from 1.6 ppm to 2.8 ppm. The lipid precursors were found in the region of 0.68 ppm and 3.27 to 3.7 ppm. The carbohydrates were identified in the region 3.3 ppm to 6.6 ppm.

Multivariate data analysis

Due to the high dimensionality and complexity of NMR data, PCA and PLS-DA approaches were employed to investigate dispersion in the metabolic datasets and to identify correlated groups and outliers. The PCA analysis with three principal components accumulated an overall 78.6% variance explained while Principal Component 1 (PC1) contributing the maximum variation of 41%, PC2, PC3 explained 19.8, 17.8% of the total variance, respectively. The results of the score plot clearly showed that the data from six groups were separated into three groups (Fig. 1a). Samples from the same groups were accumulated and the metabolites from TR1 were mainly separated by PC1. The metabolic profile of transgenic lines TR2, TR3, TR4, and TR5 were more correlated, except the TR1 line which was negatively influenced by PC1 while WT was separated on to positive PC2. The loading plot was used to analyse distinct metabolites responsible for the variable separation. The loading plot revealed that 2-PG DHA, malate, glucose 6-P, glucose-1-P, malate, and trigonelline as outliers (Fig. 1b).

Supervised multivariate data analysis, PLS-DA approach was applied for better discrimination among samples. The PLS-DA analysis showed a separation pattern similar to PCA analysis (Component 1, 37%; Component 2, 19.3%; Component 3, 14% variance). The validation cross-validation analysis was also performed for the PLS-DA model and the high Q^2 value (0.82 > 0.5) indicated a good predictive ability of PLS-DA model (**Supplementary Fig. 2a**). The score plot between component 1, component 2, and component 3 and the loading plot for PLS-DA analysis was represented in **Supplementary Fig. 2b, 2c.** The loading plot is preferably sorted by size to separate up and down-regulated metabolites. As shown in **Supplementary Fig. 2c**, the extracts from leaves of *Jatropha* were characterized as having a lower level of glucose, fructose, pyruvate, fumarate, glycerol, and the higher level of malate, glucose 6-P, choline, DHA, and AC.

Supervised OPLS-DA was used to visualized differences between transgenic lines (TR1-TR5) and their wild-type (WT) counterparts. OPLS-DA model of NMR data revealed significant differences between TRs and WT control samples in terms of primary and secondary metabolites (Fig. 2a, c, e, g and **i**). To identify significant metabolites that changed significantly in TR compared to WT samples were compared based on the S-plot from OPLS-DA. The S-plot helped to identify the metabolites that change significantly (*p*-value < 0.05) between TR and WT. Identified metabolites in transgenic lines, changed significantly appear farthest from the origin (Fig. 2b, d, f, h, and **j**). Using VIP scores, we identified ten statistically significant metabolites by each method. The variable influence on projection (VIP) value > 1 is considered important towards the observed results. Based on VIP score, lipid2, trehalose, glucose 6-P, fructose, DHA, fructose, glucose 1-P, and glycerol were significantly influenced the discrimination of all samples (**Supplementary Fig. 1)**. The metabolites qualifying the selection criteria of at least two of the above approaches were listed in Table 2. The analysis with *t*-tests showed that in the transgenic leaves, a total of 31 metabolites identified, which showed significant changes when compared to wild type control samples (**Supplementary.** Table 1). The highest number of metabolites that showed significant changes was seen between WT-TR1 and WT-TR4 samples followed by TR3, TR2, and TR5 respectively.

Metabolite profiling by H-NMR spectroscopy

The metabolome of the transgenic lines underwent significant intended and unintended changes as compared to the wild type. We observed more accumulation of lipid content in transgenic lines as compared to the wild type. The precursors of glycerophospholipids like PC, GPC, and PEA, were increased in transgenic lines **(**Table 1**)**. Also, sterols like cholesterol which is a major component of the membrane and surface lipid of leaves seemed to increases significantly. The enhanced accumulation of triglycerides due to over-expression of the *AtDGAT1* gene might also have contributed to the enhanced lipid signals.

Amongst the carbohydrates, glucose was found to be elevated by more than two-fold in the transgenic lines. A decreasing trend was observed for glucose 6-P levels, which is an intermediate in gluconeogenesis. Glucose 1-P, the key component of starch metabolism was significantly high in all the

transgenic lines. Sucrose which is a predominant product of photosynthesis was increased up to two-fold. Other sugars like fructose, cellobiose, and trehalose also showed a similar pattern. Pyruvate and glycerol derivatives like glycerate and 2-PG increased significantly in some transgenic lines. The drastic reduction in the levels of DHA suggested its consumption in pyruvate or glucose synthesis.

Moreover, the TCA cycle intermediates were increased disproportionately in the transgenic lines. Pyruvate, citrate, and fumarate were increased but malate was found to be reduced significantly in all the transgenic. This might be due to the conversion of malate to pyruvate directly, bypassing the formation of oxaloacetate. The NADPH produced in this step might be shuttled to compensate for the energy imbalance due to altered fatty acid and lipid metabolism. The unintended increase in trigonelline content may be induced by the potential increase in the nicotinamide content in the form of $NADP^+$/NADPH or NAD^+/NADH as a result of altered energy pathways [35, 36].

Metabolite-metabolite correlation and classification of transgenic lines

From the correlation studies shown in, it was evident that sugars like glucose, fructose, sucrose, cellobiose, and trehalose were positively correlated, indicating a proportionate increase of carbohydrate content in the transgenic lines. Pyruvate, citrate, and fumarate were positively correlated showing an increasing pattern, except the malate, which is decreased in all the transgenic lines. This indicated accumulation of TCA cycle intermediates due to alteration in the rate-determining step of malate to oxaloacetate conversion. Malate, DHA, and choline were negatively correlated to metabolites except for glucose 6-P and *O*-acetylcholine. This suggested that the increment observed in the accumulation of these metabolites might be compensated by the enhanced consumption of malate, DHA, and choline to maintain the energy balance (Fig. 3a**)**.

The ^{1}H-NMR analysis revealed that the metabolome of all the transgenic lines got altered following genetic manipulation resulted from the overexpression of the *AtDGAT1* gene. But the alteration was not similar among different transgenic events. The heatmap shown in Fig. 3b, classified the transgenic lines in different clades based on the similarity in their metabolome. For instance, in the line TR1, the overall increase in the carbohydrates is insignificant, as compared to the lines TR2, TR3, TR4, and TR5 where the carbohydrate store was significantly expanded. Similarly, the fatty acid accumulation was enhanced in TR1, but the change in other transgenic lines could not be claimed due to statistical insignificance. Also, the lipid content and some lipid precursors seemed to remain unaltered in the line TR3. For biodiesel production aspects, TR2, TR4, and TR5 seemed to be more suitable as they have enhanced lipid content as well as carbohydrate store availability for lipid conversion.

Pathway Analysis

Metabolic pathway analysis was performed using the web tool MetaboAnalyst 5.0 to determine the impact of metabolites on various pathways due to any change in the number of their key players, we calculated the fit coefficient (*p*) and impact factor (**Xia et al., 2015**). The pathways with an impact value ≥ of 0.1 were considered to be significantly changed **(**Fig. 4a; Table 2**)**. For instance, starch and sucrose metabolism with an impact value of 0.538, predicted to be affected the most due to alteration in the amount of the four key metabolites matched with the highest fit coefficient (glucose, glucose 1-P, sucrose, and trehalose) (Fig. 4a). The TCA cycle was predicted to be impacted significantly due to changes in the accumulation of pyruvate, citrate, malate, and fumarate. Also, the glycerophospholipid metabolism was altered due to the change in the precursors like choline, PC, GPC, and PEA (Fig. 4a).

To further examine the role of *Arabidopsis DGAT1* at the metabolic level, we analysed the metabolite profile of *DGAT1* transgenic lines. Overexpression of *DGAT1* had mainly affected the leaf metabolite profiles relative to control plants. Among a total of 31 metabolites were identified by 1H-NMR, including 15 up and 4 down-regulated in *DGAT1* transgenic lines. Based on the metabolite pathway analysis the most enriched pathways from the 31 metabolites were starch and sucrose metabolism followed by TCA cycle, galactose metabolism, and glycerophospholipid metabolism (Fig. 4a & 4b). The concentration of most significant metabolites from starch and sucrose and metabolism and TCA cycle intermediates were shown in Fig. 5. Metabolites from starch and sucrose metabolism were found to be increased in all transgenics compared to wild-type. Malate was lower in transgenics however citrate and fumarate were increased in all transgenics. All identified metabolites were also compared among transgenic lines and wild-types using Tuke's multiple comparison test (**Supplementary Table 1**). Present study has suggested that the significant changes in metabolites were followed by insertion of *Arabidopsis* DGAT1 enzymes in transgenic Jatropha.

Table 2
Metabolic pathways constituting the identified metabolites and their impact.

S.N.	Pathway Name	KEGG ID	Metabolites	*p*-value	FDR	Impact
1	Starch and sucrose metabolism	00500	Glucose	0.000	0.008	0.538*
			Glucose 1-Phosphate			
			Sucrose			
			Fructose			
			Trehalose			
2	Citrate cycle (TCA cycle)	00200	Pyruvate	0.000	0.011	0.178*
			Citrate			
			Malate			
			Fumarate			
3	Galactose metabolism	00052	Sucrose	0.001	0.022	0.058
			Glucose			
			Glucose 1-Phosphate			
			Glycerol			
4	Glycerophospholipid metabolism	00564	O-Phosphoethanolamine	0.008	0.170	0.166*
			Choline			
			Phosphocholine			
5	Amino sugar and nucleotide sugar metabolism	00520	Glucose 1-Phosphate	0.030	0.353	0.111*
			Fructose			
			N-acetyl glucosamine			
6	Glyoxylate and dicarboxylate metabolism	00630	Citrate	0.032	0.353	0.109*
			Malate			
7	Carbon fixation in photosynthetic organisms	00710	Malate	0.048	0.414	0.094
			Pyruvate			
8	Pyruvate metabolism	00620	Pyruvate	0.048	0.414	0.322*
			Malate			

S.N.	Pathway Name	KEGG ID	Metabolites	*p*-value	FDR	Impact
9	Alanine, aspartate and glutamate metabolism	00250	Pyruvate	0.052	0.414	0.006
			Fumarate			
10	Glycolysis or Gluconeogenesis	00010	Pyruvate	0.066	0.446	0.109*
			Glucose-1 phosphate			
11	Sphingolipid metabolism	00660	Phosphoethanolamine	0.202	1.000	0.033

Conclusions

^{1}H-NMR-based metabolomics determined significant variation in the metabolomics profile of Jatropha following *AtDGAT1* insertion. The analysis revealed expansion of the carbohydrate and lipid stores following genetic modification. The proportionate and disproportionate impacts in the pathway were observed. There seemed to be a major alteration on the energy pathways to compensate for the TAG accumulation in the transgenic plants, but none of the alterations seemed to affect the plant growth adversely. The present findings exhibit that plant metabolomics can play an important role in functional genomics. Present work indicates that the metabolomics approach is potentially useful to identify the metabolic differences among the various level of genetic modification. Overall, NMR profiling provided a rapid, unbiased, and reliable method for the assessment of alterations in all abundant proton-containing metabolites. Differences between the abundance of fatty acid, lipid derivatives, and carbohydrates in the transgenic lines and wild-type Jatropha were readily visible by this technique. Understanding the intended and unintended changes in the primary metabolism due to genetic manipulation provides important information for the improvement in the development of new genetically engineered plants for fatty acid and lipid accumulation. This would offer more economic benefits for biodiesel production worldwide with engineered Jatropha enhancing the sustainability for its cultivation globally.

Declarations

Ethics Approval

Since this study does not involve use of any animal, human being or cell line as such, there is no ethical issue.

Consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interest.

Funding

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Author Contribution

Conceptualization: Devendra Kumar Maravi, Lingaraj Sahoo, Vaibhav V Goud. Original draft preparation and Writing: Devendra Kumar Maravi, Lingaraj Sahoo. Review and editing: Lingaraj Sahoo, Vaibhav V Goud, Nozomu Sakurai and Ajay Kumar Biswal.

Data availability

All data generated or analyzed during this study are included in this manuscript.

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Table

Table 1 is available in the Supplementary Files section.

Figures

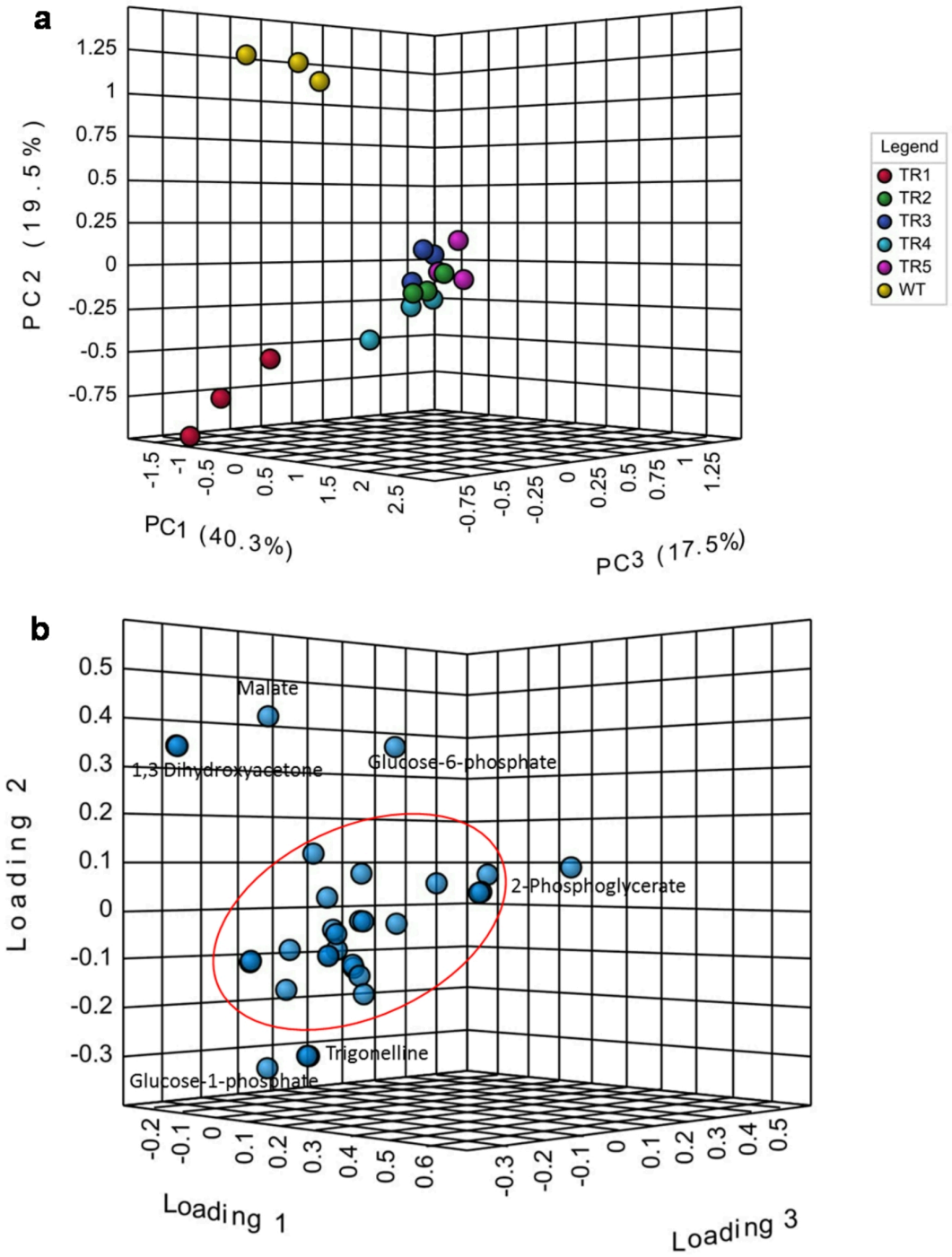


Figure 1

Principal component analysis (PCA) score plot **(a)** and loading plot **(b)** demonstrating the metabolites changes between transgenic lines and wild-type control.

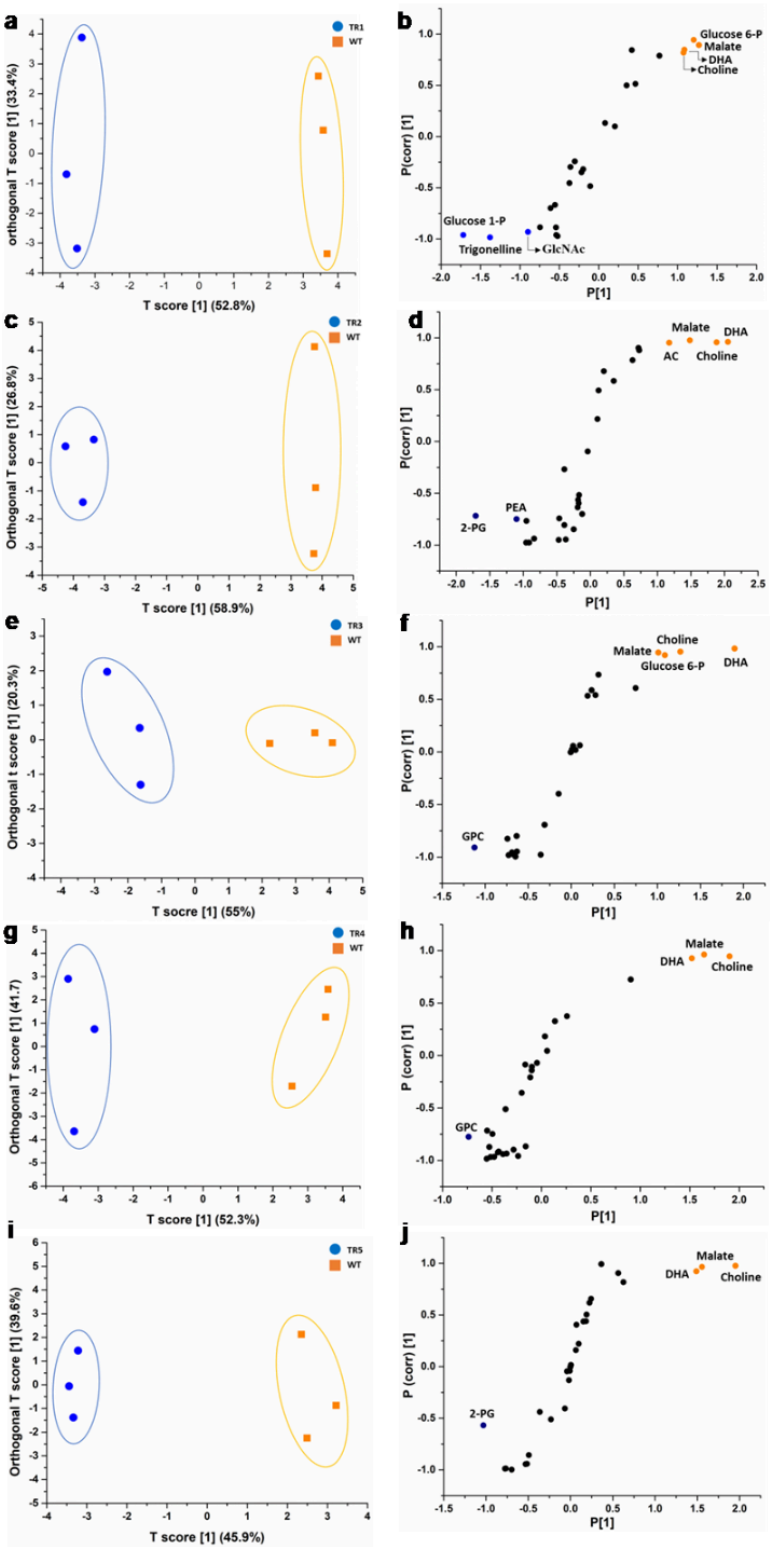


Figure 2

Score plot (**a, c, e, g** & **i**) and corresponding S-plot (**b, d, f, h** & **j**) from the OPLS-DA model between WT and TR groups.

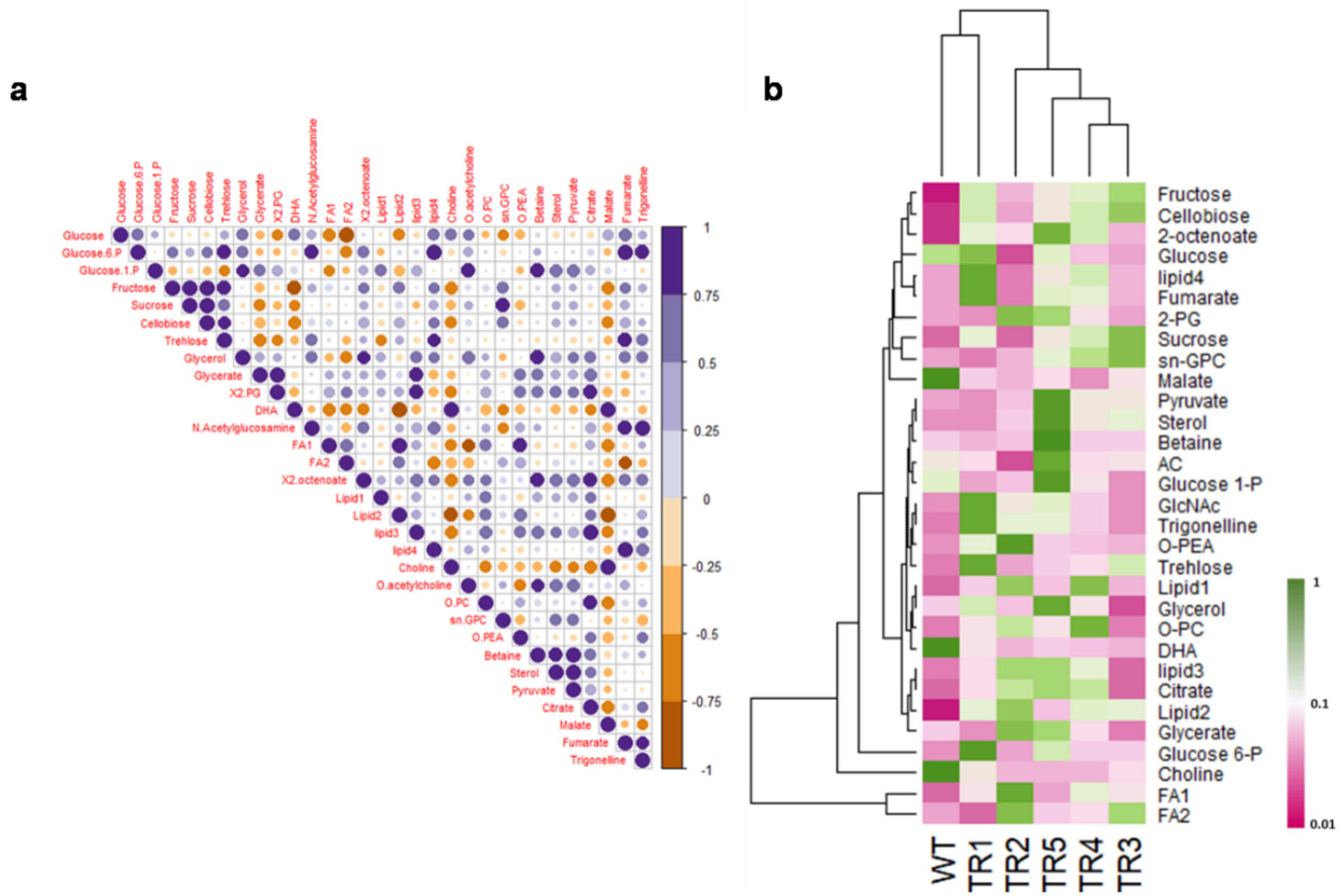


Figure 3

Metabolite-metabolite correlation (**a**) and Hierarchical clustering and heat-map (**b**)

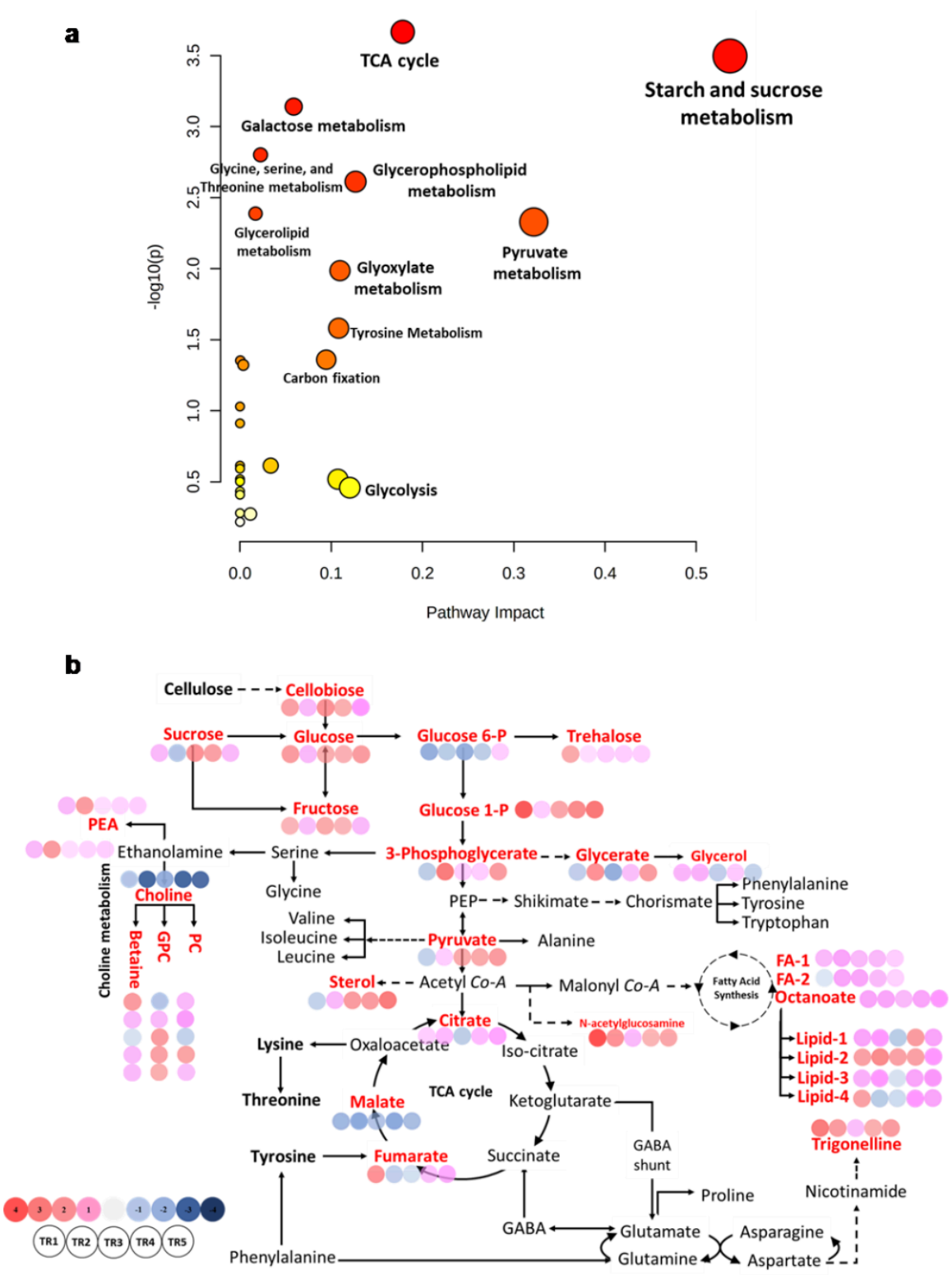


Figure 4

The metabolic pathway analysis of the model separated metabolites indicated remarkable differences among transgenic lines (**a-b**).

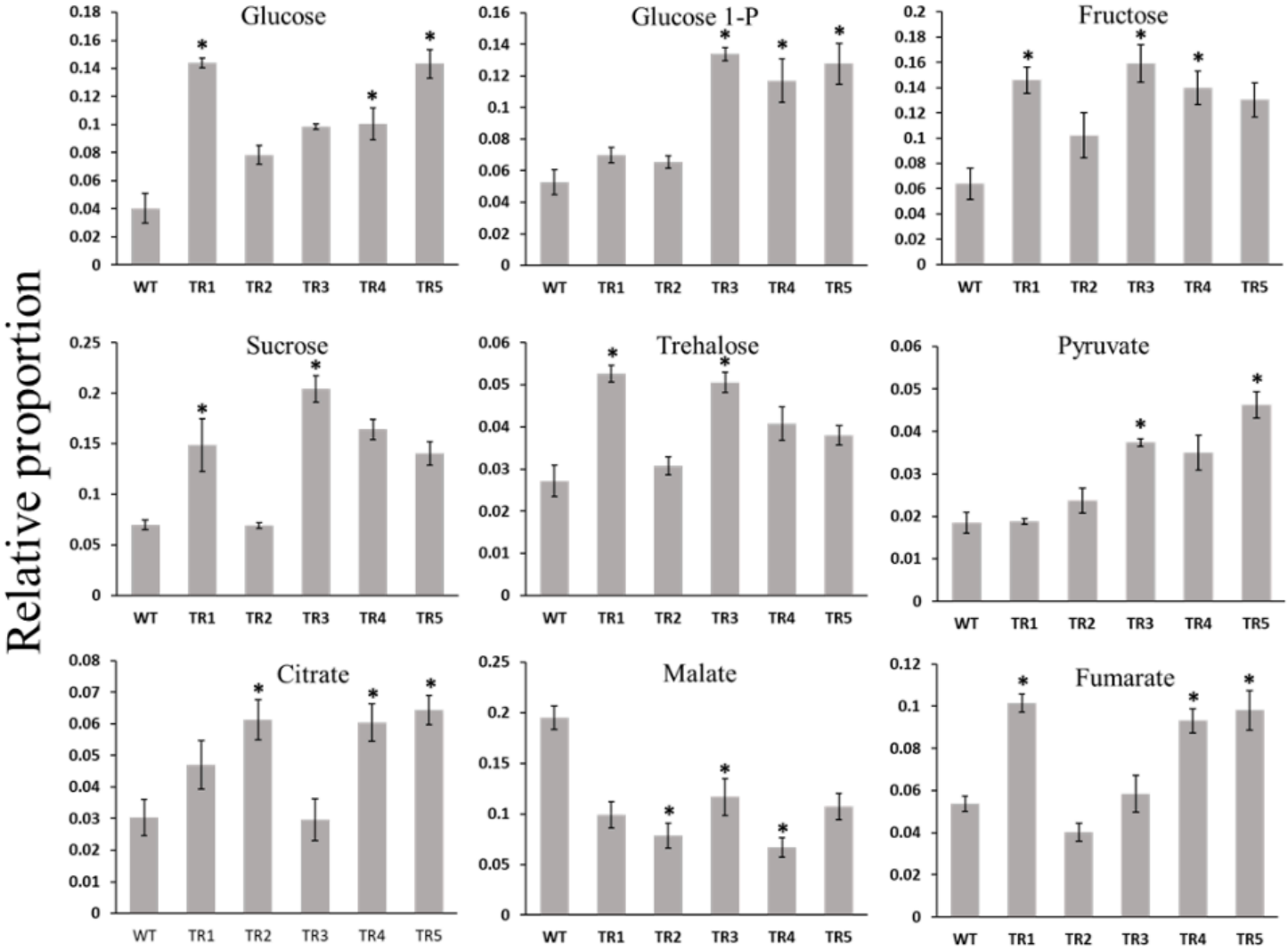


Figure 5

The relative proportion of metabolites identified in pathway analysis. (*) The asterisk represents significant differences among samples ($p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- Supplementary.docx