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# Investigation of Possible Changes Induced by RNA Silencing in Some Leaf Metabolites of Transgenic Sugar Beet Events

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ABSTRACT

Sugar beet is vulnerable to rhizomania as the most destructive viral disease. Two selected events of transgenic sugar beet carrying cassettes inducing RNA silencing mechanism, 219-T3:S3-13.2 (S3) and 6018-T3:S6-44 (S6), were shown to inhibit propagation of *Beet Necrotic Yellow Vein Virus*, the causative agent. As a method for signifying the substantial equivalence, we analyzed the levels of some metabolites through LC-MS in order to demonstrate possible unintended changes in the leaves of the transgenic events. There was no significant difference in the concentrations of examined key metabolites but *cis*-aconitate and fructose-1,6-bisphosphatase which were decreased in S3. Also, ATP was reduced in both genetically modified sugar beets. Among free amino acids, only glycine level in S6 was increased compared to the wild plant, while the production levels of 5 and 12 ones were increased in S3 compared to S6 event and the wild type plants, respectively.

## 1. Introduction

Although the sustainable development of genetically modified (GM) crops is essential for food security, still risk assessment of them is an important issue because of the unpredictable insertion of introduced gene(s) that might cause unexpected effects on metabolism changing metabolite concentrations (Kamle and Ali, 2013; Gong and Wang, 2013). This is because it is presumed that a GM crop is an organism in which the cell contents may have changed in a manner that may not happen naturally (Kamle and Ali, 2013). Therefore, it is necessary to compare the molecular properties of GM crop with non-GM parental plants for detecting these potential unexpected effects and ensuring the biosafety of GM crops in the framework of substantial equivalence concept (Baker et al., 2006). Of course, the mere difference between them is not a reason for the unsafety of transgenic crops. However, the changes must be in the range of natural alterations and should not lead to the generation of new toxic or allergenic compounds in particular (Herman and Price, 2013).

Metabolomics allows detecting the effects of genetic engineering on metabolites profiles by using several approaches mainly based on mass spectrometry (MS). The resulting data could be useful for metabolites comparisons between GM crops and their non-GM parental plant (Simó et al., 2014).

Significant improvement in resistance against rhizomania, a challenging viral disease, in sugar beet (*Beta vulgaris*), an important industrial crop, has been achieved by intensive crop breeding and also genetic engineering. Rhizomania is caused as a result of infection by *Beet necrotic yellow vein virus* (BNYVV) activity. BNYVV is widespread in most sugar beet-growing fields around the world. The virus genome is multicomponent; comprising of four or five single-stranded RNAs. To date, four types of the virus have been described. Constriction of tap root and proliferation of rootlets leading to reduced sucrose storage, are symptoms of this serious global disease (Galein et al., 2018). Recently, we have developed two types of rhizomania-resistant transgenic sugar beet plants, S3 and S6 events, through promoted RNA silencing against BNYVV. A sequence-specific RNA degradation mechanism is part of the natural plant immune system against the viral infection. As a result, virus resistance mediated by this mechanism is extremely specific and presumed to be safe for food, feed, and environment, since no ectopic protein is expressed (Zhou, 2012). Our attempt in the present study was to compare the accumulation profiles of some sugar beet metabolites to find out the possible effects of transgenesis and also RNA silencing

*Abbreviations:* GM, genetically modified; MS, Mass spectrometry; BNYVV, *Beet necrotic yellow vein virus*.

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mechanism. The presented data improve our insight about differences in the metabolome profiles of the transgenic sugar beet with their parent one.

## 2. Materials and methods

### 2.1. Plant cultivation and harvesting

Two transgenic sugar beet (*Beta Vulgaris* L.) events, 219-T3:S3-13.2 or IHP-P (S3) and 6018-T3:S6-44 or IHP-U (S6), were already developed in National Institute of Genetic Engineering and Biotechnology (Zare et al., 2015). The wild type parental plants, Var. 9597 (WT), were obtained from Sugar Beet Seed Institute. Virus resistance was attributed to both events S3 and S6 by two different constructs; IHP-P carried two copies of 5'-UTR of RNA2 with the gene sequence encoding P21 protein and IHP-U has only two copies of 5'-UTR, which were positioned in the sense and antisense with an intron expressing hpRNA in the middle of the construct (Zare et al., 2015). The seeds were sown in the plastic cup containing equal parts of autoclaved soil and sand, and maintained in a growth room with a 16h/8h light/dark cycle, at 25 °C/20 °C day/night temperature, and a relative humidity of 60%. At eight weeks post-germination, plants were transferred into 1L plastic pots containing the same soil/sand composition and were arranged in a completely randomized design with four replicates in a growth room with the above conditions. After three months, leaves were harvested directly into liquid nitrogen and either freeze dried or stored in -70 °C.

### 2.2. RNA extraction and comparative RT-PCR

Total RNA was extracted from the BNYVV-infected plants using RNX-Plus kit (Sinaclon, Tehran, Iran) followed by DNase treatment. cDNA was synthesized using first strand cDNA synthesis kit (Sinaclon, Tehran, Iran). RT-PCR reaction was done by the first cycle at 93 °C for 6 min, being followed by 35 cycles at 93 °C for 30 sec, 65 °C for 45 sec, 72 °C for 1 min and a last cycle at 72 °C for 10 min. Then, the PCR products were visualized by agarose gel electrophoresis.

### 2.3. Extraction and quantification of metabolites

For the selected metabolites, 20 mg freeze-dried material was used for extraction of metabolites using chloroform-methanol. After extraction, samples were dried and resolved in ultra-purified (UPLC) water. The analysis was performed on liquid chromatography connected to mass spectrometer (LC-MS). Final volume for the samples was 300 µl. For the calculation of the metabolite concentration, a mixture of external standards with 30 different metabolites and 6 different concentrations were used. The standard mixtures were measured three times during the run, at the beginning, in the middle, and at the end. The calibration curves were prepared for all the measured metabolite standards.

### 2.4. Extraction and quantification of amino acids

10 mg freeze-dried material was dissolved in 400 µl UPLC water. Individual samples were subjected to a derivatization procedure using IPK-produced fluorescing compound (ACQ) and used for UPLC separation. Derivatization was carried out with 10 µl sample in 90 µl of UPLC water. 1 µl of derivatized samples were loaded onto the column of ultra pressure reversed-phase chromatography (UPLC). The UPLC system consisted of a quaternary solvent manager, a sample manager-FTN, a column manager and a fluorescent detector (PDA eλ Detector). Separation was carried out on a C18 reversed-phase column (Luna Omega, 1.6 µm, 2.1x100 mm, Phenomenex, Germany) with a flow rate of 0.6 ml per min and a duration of 6 min. The column was heated at 45 °C during the whole run. Detection wavelengths were 266 nm for excitation and 473 nm for emission. The gradient and the corresponding eluents were accomplished according to the protocol of Bioanalytics Gatersleben, Germany. For the calculation of final concentrations, external standard mixtures with 6 different concentrations were used. The standard amino acids mixtures were measured three times during the run, at the beginning, in the middle, and at the end. The calibration curves have been prepared from all the measured standards. Single standards for all amino acids were used to verify the correct retention times. The quantification was carried out based on external standards with various concentrations.

### 2.5. Calculations and statistical analysis

For standardizing each metabolite data, the measured content value was multiplied by the total extract volume (400 µl) and divided by the dry weight. The analysis of the differences among means of metabolites and free amino acids levels was conducted with Duncan's method at confidence interval of $P < 95\%$.

## 3. Results

This study presented data for investigating the substantial equivalence of the third generations of two homozygous GM sugar beets in comparison with their parental plant grown in the same conditions by measuring the levels of free amino acids and some key metabolites. Having confirmed the presence of transgene in the chosen events (Hejri et al., 2021), half of the plants at six leaf stages were challenged with the virus for two months. The viral propagation inhibition by RNA-silencing mechanism against P21 of BNYVV in the transgenic events was reaffirmed by comparative RT-PCR. The reduced level of amplified P13-encoding gene indicated the levels of inhibition of viral propagation in the wild type or S3 and S6 transgenic events as expected (Fig. 1). The levels of free amino acids and the selected metabolites in four biological replicates for each unchallenged plants leaves were quantified via LC-MS to evaluate the possible differences due to presence of transgene and/or induced RNA silencing mechanism. The means values are presented in Tables 1 and 2 for the selected metabolites and all free amino acids, respectively.

There was no significant difference in the concentrations of malate, succinate, *trans*-aconitate, citrate, and fumarate between the three plants. In addition, in genetically modified sugar beets, glucose, fructose, sucrose, glucose 1-phosphate (Glc1P), glucose 6-phosphate + fructose 6-phosphate (Glc6P + Fru6P), ADP-glucose (ADPGlc), UDP-glucose (UDPGlc), 3-phosphoglycerate (3PGA), and phosphoenolpyruvate (PEP) were maintained at the same levels as those in the parental one. Glc6P and Fru6P could not be separated and were thus calculated together. While the amounts of most energy carrier compounds, like ADP, AMP, UTP, UDP, and UMP, remained stable, the level of ATP in the

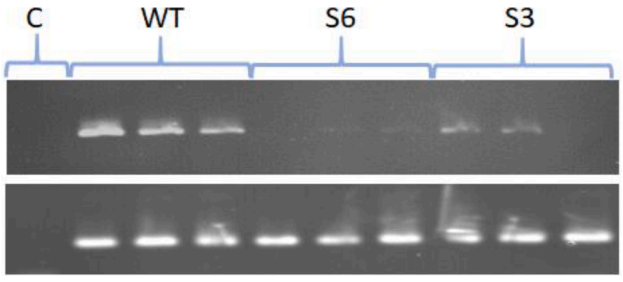


**Fig. 1.** Viral propagation inhibition by RNA-silencing mechanism in the transgenic events. RT-PCR products of expressed plant actin gene, as the internal control (bottom panel, 200-bp bands), and viral P13 gene (top panel, 220-bp bands) in wild type (WT) or transgenic events (S3 and S6) challenged with BNYVV for two months were separated on agarose gel and stained with ethidium bromide. Three biological replicates for each plant type were used. Lane C, technical control with no cDNA added.

**Table 1**
Comparing the contents of some metabolites (μmol $g^{-1}$DW for glucose, fructose and sucrose and nmol $g^{-1}$DW for the others) related to sucrose metabolism or glycolysis and TCA cycles in the transgenic and wild type plants.

| S6 | S3 | WT | Metabolite |
|---|---|---|---|
| 79.060 A | 62.980 A | 68.228 A | Glucose |
| 41.460 A | 36.665 A | 39.850 A | Fructose |
| 15.660 A | 18.300 A | 13.811 A | Sucrose |
| 3.623 A | 3.500 A | 4.420 A | AMP |
| 1.440 A | 1.058 A | 1.248 A | ADP |
| 0.325 B | 0.340 B | 1.083 A | ATP* |
| 11.893 A | 0.650 A | 2.033 A | UTP |
| 0.185 A | 0.145 A | 0.643 A | UDP |
| 0.660 A | 0.565 A | 0.493 A | UMP |
| 5813.875 A | 5846.675 A | 5520.275 A | Malate |
| 33.638 A | 32.175 A | 14.208 A | Succinate |
| 53.713 A | 48.328 A | 56.123 A | Trans-aconitate |
| 3090.875 A | 3564.725 A | 4123.500 A | Citrate |
| 20.770 A | 51.158 A | 33.258 A | Fumarate |
| 4.570 A | 3.045 A | 2.575 A | Glc1P |
| 4.808 A | 1.963 A | 1.808 A | Glc6P + Fru6P |
| 0.778 A | 0.910 A | 0.983 A | 3PGA |
| 0.088 A | 0.068 A | 0.278 A | PEP |
| 0.010 A | 0.006 A | 0.010 A | ADPGlc |
| 0.368 A | 0.235 A | 0.438 A | UDPGlc |
| 29.533 AB | 24.788 B | 48.980 A | Cis-aconitate* |
| 30.240 AB | 24.188 B | 43.500 A | Fru1,6bisP* |

*Metabolites with significant differences in concentration means among three types of plants at $P < 0.05$.

**Table 2**
Concentrations of free amino acids (nmol $g^{-1}$DW) in the transgenic S3 and S6 vs. wild type plants.

| S6 | S3 | WT | Amino acid |
|---|---|---|---|
| 16.549 A | 17.412 A | 3.617 A | Arg |
| 116.861 A | 174.563 A | 125.262 A | Ser |
| 713.986 A | 892.716 A | 620.221 A | Glu |
| 136.257 A | 145.880 A | 131.218 A | Gaba |
| 39.863 A | 110.230 A | 33.878 A | Pro |
| 17.969 A | 14.578 A | 18.395 A | Tyr |
| 6.654 A | 5.716 A | 8.656 A | Met |
| 173.706 A | 188.839 A | 129.001 B | Gly* |
| 122.035 B | 225.190 A | 115.939 B | Asn* |
| 123.964 B | 293.509 A | 134.437 B | Gln* |
| 9.526 B | 15.840 A | 6.577 B | Asp* |
| 36.098 B | 68.828 A | 36.820 B | Thr* |
| 133.907 B | 202.568 A | 134.912 B | Ala* |
| 30.707 B | 57.318 A | 20.344 B | Val* |
| 8.289 AB | 10.381 A | 5.488 B | His* |
| 12.856 AB | 17.842 A | 10.436 B | Lys* |
| 21.026 AB | 35.227 A | 14.815 B | Ile* |
| 19.730 AB | 38.479 A | 11.399 B | Leu* |
| 10.841 AB | 18.695 A | 8.828 B | Phe* |

*Amino acids with significant differences in concentration means among three types of plants at $P < 0.05$.

wild type plants was three times higher than the level of ATP in the transgenic events. Also, the levels of fructose-1,6-phosphate (Fru1,6-bisP) and *cis*-aconitate were altered in S3 and reduced by 2-fold compared to non-GM type (Table 1). In practice, the levels of iso-citrate, pyruvate, acetyl-CoA, oxoglutarate, sucrose 6- phosphate, trehalose 6- phosphate, and glucuronic acid were under detection limits in the samples.

There was no significant difference in total free amino acid contents between S6 event and parental wild type (1750.824 nmol $g^{-1}$DW vs. 1570.233 nmol $g^{-1}$DW), while this value in S3 event (2533.811 nmol $g^{-1}$DW) was 1.6 times higher than non-GM one.

As shown in Table 2, like other plants (McCusker et al., 2014; Kumar et al., 2017), glutamate (Glu) was the most abundant free amino acid in all sugar beets, while methionine (Met) and histidine (His) were the least abundant in transgenic and wild type plants, respectively. There were no statistical differences in the concentration means of arginine (Arg), serine (Ser), Glu, GABA, proline (Pro), tyrosine (Tyr) and Met between the three types of plants. Changes in the amounts of glycine (Gly) occurred in both transgenic plants. Both transgenic sugar beets exhibited higher concentrations of Gly (188.839 and 173.706 nmol $g^{-1}$DW for S3 and S6, respectively) compared to the wild type one (129.001 nmol $g^{-1}$DW). For S3, increases in the amounts of His, lysine (Lys), isoleucine (Ile), leucine (Leu), and phenylalanine (Phe) rather than the control plant were notable. For instance, the concentration of Leu was approximately tripled in S3 in comparison with the wild type plant. Also, significant differences of the concentrations of glutamine (Gln), aspartate (Asp), asparagine (Asn), threonine (Thr), alanine (Ala), and valine (Val) were found between the S3 versus control or S6 plants. For example, the amount of Gln in S3 was more than twice higher than the other two plants.

## 4. Discussion

Genetic manipulation for a target gene may affect the expression of other genes, thus, altering the concentrations of metabolites that are irrelevant to the primary purpose of introducing a new trait. One procedure to evaluate genetically modified plants safety is the comparison with the plants for which the safety history of their uses is available, parental wild type plant in particular. In the present research, free amino acids and some key metabolites profiles of two transgenic sugar beet events, S3 and S6, were compared with the parental plant pairwise.

### 4.1. Selected key metabolites

While no significant differences were detected for 19 metabolites contents, the levels of Fru1,6bisP and *cis*-aconitate decreased in S3. Cis-aconitate isomer is generated from citrate and then converted to iso-citrate in the citric acid cycle (Taiz and Zeiger, 2006). Fru1,6bisP is involved in the sucrose metabolism which is mainly limited to oxygenic photosynthetic organisms. As the core product of photosynthesis in higher plants, sucrose moves between plant tissues and plays a central role in growth and development, storage, signal transduction, and adaptation to environmental stresses (Salerno and Curatti, 2003). During sucrose biosynthesis, triose phosphate is firstly produced in the chloroplast through Calvin cycle, and transported to the cytosol by triose phosphate/phosphate transporter where two triose phosphates are converted to a Fru1,6bisP molecule by aldolase activity. Afterwards, hexose phosphates like Fru6P, Glc6P, Glc1P, and UDPGlc are generated. UDPGlc, in combination with Fru6P, makes sucrose-6-phosphate, which is then converted to sucrose by the activity of sucrose phosphate phosphatase (Salerno and Curatti, 2003; Stein and Granot, 2019). In a reverse pathway, Fru1,6bisP is formed which enters the glycolysis pathway and can be broken down into triose phosphates that eventually turn to pyruvate in a multi-step process. Pyruvate can enter various pathways in the cell. For example, it can enter the TCA cycle in the presence of oxygen, the production of fatty acids or amino acids (Taiz and Zeiger, 2006). Decreased Fru1,6bisP in S3 event can affect the sucrose synthesis and glycolysis pathways, and consequently the TCA cycle. However, no changes in the sucrose and PEP contents at the end of the sucrose synthesis and glycolysis pathways, respectively, indicated that there was probably no alteration in both routes. This is also supported by unchanged levels of UDPGlc, Glc1P, fructose and Glc6P + Fru6P for sucrose synthesis way as well as citrate, succinate, fumarate, and malate for glycolysis pathway and TCA cycle. Therefore, it is possible that the glycolysis components, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, which lead to the biosynthesis of pyruvate in a multi-step process, or components of the sucrose generation pathway were provided through other ways such as Calvin cycle and starch degradation, respectively. It is possible that the expression of phosphofructokinase (a committing enzyme for Fru1,6bisP biosynthesis) or aldolase (an enzyme that converts Fru1,6bisP to triose

phosphates) were affected by gene insertion. However, the lack of Fru6P accumulation weakens this presumption that phosphofructokinase was affected by genetic engineering. Nevertheless, it is important that the sucrose content did not change in both transgenic events, because the main economic importance of sugar beet is due to its high sucrose content.

The amount of ATP decreased approximately 3-fold in transgenic events. ATP produced during photosynthesis in chloroplast and respiration in mitochondrion functions as a carrier of energy in metabolic processes by storing energy in its phosphate-phosphate bonds, therefore, plays an important role in energy transfer. It is a precursor to nucleic acids and can also act as a coenzyme in some reactions (Dunn and Grider, 2020). Also, extracellular ATP acts as a signal molecule (Tanaka et al., 2010). Scientific evidences suggest that ATP is highly required for two steps in RNA silencing mechanism, dsRNA processing to siRNA and the unwinding two siRNA strands. Also, it is required for maintaining 5 ’phosphates on siRNAs (Nykänen et al., 2001). The consumption of ATP in RNA silencing mechanism may explain the significant reduction of ATP levels in both transgenic events. In addition, the level of His, whose biosynthesis from phosphoribosyl pyrophosphate consumes a large amount of ATP (Galili et al., 2016), has also increased in the S3 transgenic plant.

### 4.2. Free amino acids

Amino acids serve as precursors for proteins and other metabolites such as nucleotides, phytohormones, phenolic compounds and glucosinolates (Joshi et al., 2006; Kumar et al., 2017). They are involved in many reactions and metabolic pathways in soluble forms (Azevedo et al., 2006). They play essential roles during plant growth and development, carbon and nitrogen metabolism, stress response, regulatory and signaling processes, and energy production or redox reactions (Rai, 2002; Galili, 2011; Hildebrandt et al., 2015; Kumar et al., 2017). Furthermore, free amino acids are related with pathogenesis and symbiotic interactions, particularly in rhizosphere (Kumar et al., 2017). The storage of free amino acids changes in response to either developmental and physiological stages or environmental factors (Hildebrandt et al., 2015).

Amino acid variations may be due to alterations in their biosynthesis pathways, downstream reactions or protein degradation. Amino acids can catabolize into compounds such as fumarate, succinate, 2-oxoglutarate, acetyl CoA, pyruvate, and malate, which are components of the TCA cycle, to yield energy especially under stress condition (Galili et al., 2016). Since, there was no difference in the levels of TCA cycle components between the transgenic events and the wild type plant; it is assumed that there is no change in the catabolic pathways.

Herein, the increase in amino acids whose biosynthesis is related to Asp in S3 plants is noteworthy. Asp leads to the biosynthesis of Asn, Glu, Thr, Met and Lys. Asp may also be metabolized to Gly. Ile is derived from Thr or Met. Val/Leu and Ile are generated in two separate but parallel pathways using three common enzymes. These biosynthetic routes have two common metabolites, dihydroxy methylpentanoate and methyl oxopentanoate (Fig. 2; Azevedo et al., 2006; Jander and Joshi, 2010; Joshi et al., 2010). Therefore, an enhancement of the Asp pool may have led to an increase in the synthesis pathway of all these amino acids. Yet, direct or indirect effects on the levels of His, Phe and Ala need to be explained. The increase in free Asp itself could be the result of protein degradation or oxaloacetate transamination which is produced in the TCA cycle in mitochondria or from phosphoenolpyruvate carboxylase activity in the cytoplasm. Although, oxaloacetate content was not available, the unchanged concentrations of PEP and TCA cycle components such as citrate, succinate, fumarate, and malate, which eventually convert to oxaloacetate in TCA cycle, suggest that probably oxaloacetate concentration did not change significantly in S3 either. Thus, whether

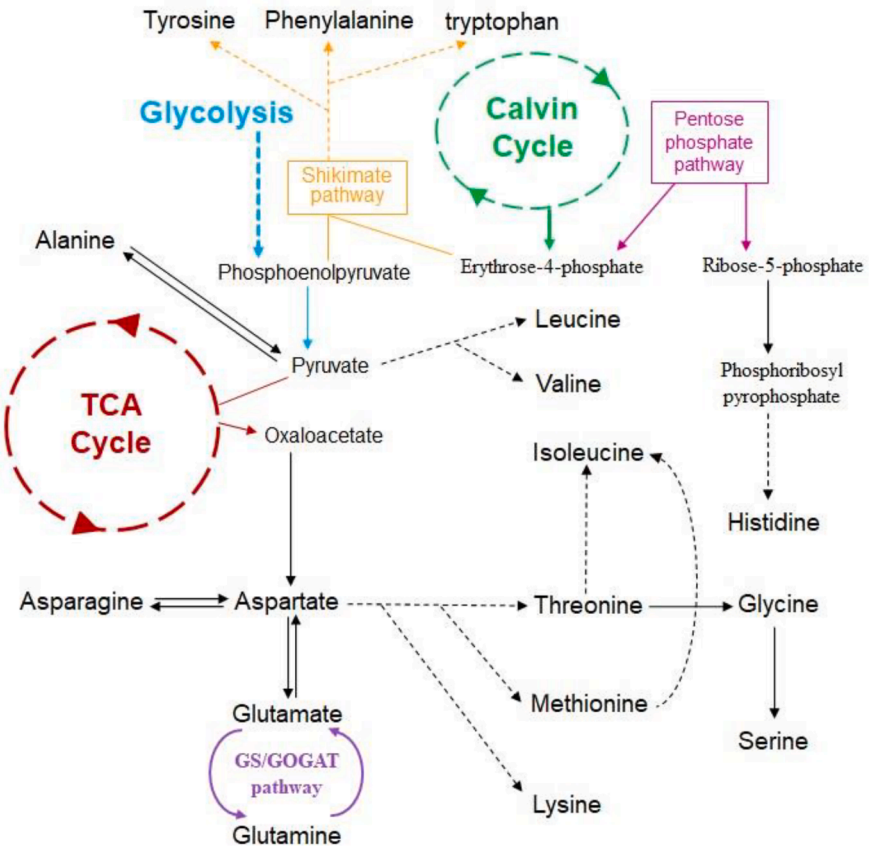


**Fig. 2.** Relationships among biosynthetic pathways of amino acids, especially aspartate.

the increases in Asp and other amino acids contents occur through the degradation of proteins or downregulation of downstream metabolic reactions need to be cleared by further analysis.

### 4.3. The extent of changes in transgenic events

In addition to simple nucleotide variations (such as mutation, insertion, and deletion), a significant portion of the genome is made up of mobile genetic elements, transposons, causing mutations and rearrangement within the genomes (Herman and Price, 2013). Besides, processes such as segmental duplication, gene transfer between organelles and nucleus, horizontal gene transfer, and other unknown possibilities lead to gross changes in plant genomes. Thus, natural diversity among individuals should be taken into account when comparing the molecular properties of transgenic plants with their wild parents (Baker et al., 2006). Therefore, it is not true to assume that all differences between GM and non-GM crops are due to genetic engineering.

Several comparative genomic studies in recent years called attention to the fact that conventional breeding, including backcrossing, mutagenesis, tissue culture regeneration, and hybridization causes extensive and diverse alterations in genomic contents of plants (Herman and Price, 2013; Stewart and Shepherd, 2013). When it comes to functional genomics, differences could also be related to not only method of transformation, gene insertion and effect of transgene expression, but also the effects of environmental and growth conditions that affect molecular composition of plants should be considered deliberately (Noteborn et al., 2000; Herman and Price, 2013; Stewart and Shepherd, 2013; Fu et al., 2019). Accordingly, what needs to be examined in these studies is whether these changes are within the range of alterations resulting from traditional breeding, natural variations, environmental conditions, or beyond them (Herman and Price, 2013).

Numerous researches suggest that gene insertion introduces less variation on the metabolome than the traditional breeding (Berberich et al., 1996; Catchpole et al., 2005; Kogel et al., 2010; Jiao et al., 2010; Corujo et al., 2019; Liu et al., 2020). For example, Fu et al. (2019) examined unexpected effects in transgenic rice using metabolomics by comparing eight varieties of GM rice and their isogenic counterparts. They found that 7 to 50 metabolites were changed at notable levels but these alterations were far fewer than that among conventional rice varieties.

The environmental factors have been revealed to play a stronger role in altering production levels of metabolites than genomic manipulation (Zhou et al., 2009; Jiao et al., 2010; Fu et al., 2019). For instance, Baker et al. (2006) tried to compare metabolome of three transgenic wheats with the corresponding parent lines planted in two fields for three years. They showed place and time of growth have a stronger effect on metabolites than genotype, and the differences between the parent lines in different places and times were greater than between the control and transgenic lines.

To know whether the observed changes in the levels of some metabolites in transgenic sugar beets occurred in the range of natural changes, studies on the metabolic profiles of sugar beet were reviewed. Hu et al. (2019) showed that the total amino acid contents of 14 sugar beet varieties ranged between 0.30% and 0.62% in root. This means that the total amount in the variety with the highest content was twice that of the variety with the lowest content. Such notable differences were also observed for each amino acid. Liu et al. (2020) presented that even one-day exposure of sugar beet to salinity increases TCA cycle activity and sucrose metabolism in sugar beet, leading to the decrease in sucrose and the increase in TCA cycle components such as malate and 2-exoglutarate. Also, after seven days, metabolites such as amino acids demonstrated considerable changes.

In our research, comparison of the S6 transgenic event versus the parent plant showed limited changes (below 5%) while these changes were significant in the S3 transgenic plant (about 35%). However, these differences were mainly in the amino acid content. In fact, the differences observed between the transgenic events and the non-transgenic plant were less than the differences observed in Hu et al. (2019) and Liu et al. (2020) researches.

## 5. Conclusion

Metabolite contents of transgenic sugar beet leaves were compared to wild type plant as a part of a risk assessment analysis. The slight alterations in S6 metabolites were expected to occur because of micro-environmental effects and/or natural individual differences. In the case of S3 sugar beet, the differences were significant but still within the natural range while being beneficial due to increased contents of amino acids, especially the essential ones. The present research demonstrates the safety of transgenic crops in which RNA silencing mechanism is exploited.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The experiments in this research were performed by independent people with no bias. Besides we have tried to present data and interoperations in a scientific manner.

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### Conflict of interest statement

Mohammad Ali Malboobi is a shareholder of Green Transgene Technology Development Co. All others have no conflict of interest.

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