Research Article

Comparative Transcriptome Analyses of a Transgenic Sugar Beet Resistant to *Beet Necrotic Yellow Vein Virus*

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Abstract

Genetic engineering is a powerful technology of the present century that has revolutionized the agricultural, health, pharmaceutical and food industries worldwide. It is important to identify changes caused by transgenes that may be attributed to unintended traits in the risk assessment of genetically modified (GM) crops. Rhizomania, which is caused by *beet necrotic yellow vein virus* (BNYVV) infection, is considered to be a significant constraint in order to produce sugar beet worldwide. The resistance of transgenic sugar beet plants to the BNYVV was previously developed through RNA silencing by expression of hairpin RNA (hpRNA) structures. In the present study, the RNA sequencing (RNA-seq) analysis was performed in order to evaluate the transcriptional changes of an event of transgenic sugar beet plants, named 219-T3:S3-13.2 (S3), with the non-transgenic parental plants grown in virus-infected soil. The results of the present study indicate that there are only 0.9% differentially expressed genes (DEGs) at significant levels. The functional analysis shows alterations of transcription in lipids, amino acids, and carbohydrates metabolisms, cellular processes (autophagy), hormone signal transduction, and biosynthesis of secondary metabolites in the transgenic event, which are related to stress-adaption for which most of the genes were up-regulated. All in all, we conclude that the presence of the transgenes does not have substantial effects on the plant gene expression patterns. This work also indicates that RNA-seq analysis can be useful to evaluate the unintended effects and risk assessment of GM sugar beet plants.

***Keywords*:** Safety assessment, RNA silencing, differentially expressed genes

Introduction

Widely acceptance and cultivation of genetically modified (GM) crops have made it the fastest-growing agricultural technology in the world. Cultivation of GM crops that are resistant to biotic and abiotic stress has benefits such as increasing farm income and crop production, as well as reducing the use of pesticides and greenhouse gas emissions (Liu and Stewart Jr, 2019). These complementary breeding techniques provide solutions for food security and climate change, and possibly introduce more desirable and wider range of food products to the market. The application of genetic engineering is only one part of agricultural innovation that contributes to the success of modern agriculture. However, like any new technology, possible risks must be assessed and managed, a task that has been left to legislators for the past 30 to 40 years (Turnbull et al., 2021).

The safety assessment of GM crops is considered based on the principle of substantial equivalence and also the analysis of comparative safety (Co-operation and Development, 1993; Kok and Kuiper, 2003; Organisms, 2011; Organization, 1996). This principle is based on the notion that a typical, almost non-GM near- isogenic type, as well as a history of safe usage can be considered as a comparator for the safety assessment of new GM crops (Benevenuto et al., 2022).

Agrobacterium-mediated transformation is one of the most common methods used to create GM crops. In the method mentioned above, transfer-DNA (T-DNA) from *Agrobacterium tumefactions* is inserted into the plant nuclear genome (Gelvin, 2017). Because of the stochastic nature of plant transformation mechanisms with *Agrobacterium*, transgenic cassettes can be integrated into genomic sites that may have unintended effects on the gene (Barros et al., 2010).

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In the recent decade, the roles of emerging "omics" technologies in the assessment of unintended transgene effects have been commonly proposed. In this regard, transcriptomics, proteomics and metabolomics are common technologies used to determine the molecular composition of a system as a measure of equivalence (Fu et al., 2021). With the advent of the next generation of sequencing and the increasing power of computing platforms, RNA sequencing (RNA-Seq or whole transcriptome shotgun sequencing) was developed and quickly replaced microarrays as the method of choice for transcriptomics with almost no bias (Almeida-Silva et al., 2021). The RNA-seq method usually involves identifying suitable biological samples (and replicates), isolating the whole RNA, enrichment of non-ribosomal RNAs, converting RNA to cDNA, building a fragmented library, sequencing on a high-throughput sequencing platform, generating single or paired-end reads with a length of 30-300 base, alignment or assembly of these reads and downstream analysis. In addition to whole transcriptome analysis, there are several ways of downstream analyses including transcript discovery and annotation, the possible gene regulation mechanisms, differential gene expression patterns, identification of alternative splicing products, allele-specific expression examination, RNA editing detection, viral detection, gene fusion detection, and other types of variant detection (Griffith et al., 2015). In particular, the RNA-Seq method facilitates the evaluation and analysis of genetic changes, mutations, and variations or differences in gene expression of different groups or treatments such as transgenic plants versus conventional plants (Matsaunyane and Dubery, 2018).

Recent advances on the next-generation of sequencing technologies along the releases of the sugar beet genome (Dohm et al., 2014) have made it possible to access more detailed information, as well as refined tools, in which they were not available before. So that, it can result in more accurate identifications of differentially expressed genes. Sugar beet (*Beta vulgaris* L. var. Saccharifera) is one of two main sugar crops worldwide that has a broad range of cultivation and significant economic values. The root is used to produce sugar, whereas the stem and leaf are used to feed and produce ethanol and biofuels (Dohm et al., 2014; Finkenstadt, 2014). Rhizomania, which is derived from the *beet necrotic yellow vein virus* (BNYVV), is considered one of the main restraints of sugar beet production in the world, so that it causes a severe reduction in sugar by 80 percent (Galein et al., 2018). The BNYVV is a member of the genus *Benyvirus* within the family *Benyviridae* (Liebe et al., 2020), a soil-borne virus transmitted by the plasmodiophorid *Polymyxa beta*. It has a multi-part genome that contains 4 or 5 positive single-stranded RNAs. Subsequently, Zare et al. (2015) have developed the BNYVV-resistant transgenic sugar beet plants and S3 event using the promoted RNA silencing versus the BNYVV by expressing the hairpin RNA (hpRNA) structures (Zare et al., 2015).

Transcriptomics approach has an important role in the assessment of potential differences between two genotypes due to the extensive coverage of plant pathways and metabolic networks compared to other "omics" approaches (Barros et al., 2010). This study focused on the transcriptomics approach of the RNA sequencing (RNA-seq) in order to identification of possible unintended effects in transgenic events. Since rhizomania virus disease significantly reduces the amount of sugar extracted from sugar beet roots and RNA silencing gets more active after soil-borne virus infection, we compared the root transcriptome profiles of wild-type (WT) sugar beet genotype 9597 (non-transgenic counterpart), as a controlling factor, with a transgenic event, which is named 219-T3:S3-13.2 (S3).

In the present study, the main hypothesis was defined that T-DNA insertion has no unintended effect on transgenic sugar beet plan. We considered probable tiny differences between transgenic sugar beet plants and their non-transgenic counterparts. The crucial question in this research is whether any difference in the transcriptome or not?

Materials and Methods

Plant Materials and Growth Conditions

The transgenic sugar beet (*Beta vulgaris* L.) event, i.e. 219-T3: S3-13.2 or IHP-P (S3), which was previously developed at the National Institute of Genetics Engineering and Biotechnology and has been performed in the present study. IHP-P carrying two copies of 5′-UTR of RNA2 with the gene sequence encoding P21 coat protein, so that they were placed in the sense and antisense connected by an intron expressing hpRNAs (Zare et al., 2015). Wild-type parental plants Var. 9597 (WT) was kindly provided by the Sugar Beet Seed Institute. Homozygous transgenic sugar beet seeds derived from the third generation and Var. 9597 (WT) seeds were planted in small pots consisting of equal amounts of autoclaved sand and garden soil at phytotron (i.e. under conditions of 16/8h light/dark, photoperiod 25/20°Cday/night and relative humidity

of 60%) in. After eight weeks of doing that step, each plant was transferred to the 1-L pot containing soil infested with the BNYVV collected from a farm around Shiraz city, Iran. All contaminated soils were diluted one to one with a 1:1:1 (v/v/v) sterilized mixture of peat, perlite, and vermiculite. Plants grew in a growing room with a light cycle of 16/8 hours of light/darkness at temperature of 25-30°C. After 3 months, the roots of plants were harvested and washed to remove the soil. The roots of plants were immediately frozen by liquid nitrogen and stored at temperature of -80°C for the RNA extraction.

RNA extraction and sequencing

The total RNA was derived from the roots of four samples including WT1 and WT2 for non-transgenic counterparts, and S31, S33 for S3 events, using the RNX plus solution according to the manufactur's instructions (SINACLON, Iran). RNA pellets were dissolved in water DEPC treated water. The total RNA was treated with DNAaseI (SINACLON, Tehran, Iran) to eliminate possible contamination of genomic DNA and then heat treatment (55°C for 10 minutes) to inactivate the enzyme and stored at temperature of -80°C. The quality of RNA was determined by the optical density (OD) ration values of OD260: OD280 and OD260: OD230, and also integrity was evaluated by 1% agarose gel electrophoresis. The RNA library and sequencing were built by the Novogene Corporation (Beijing). In summary, poly (A) $^{+}$RNA was enriched with the total RNA by oligo(dT) beads. Then, the mRNA was randomly fragmented by the Novogene Corporation (Beijing), furthermore, the cDNA was synthesized using random hexamers. The library construction includes the A-tailing, terminal repair, size selection, ligation of sequencing adapters and PCR enrichment. Paired sequencing was performed on the HiSeq 5000 Illumina platform.

RNA sequence analysis

The software "FastQC" (version 0.11.5) was utilized to control the RNA-seq data quality. Reads were cleaned using the software "Trimmomatic" (version 0.39; http://www.usadellab.org/cms/?page=trimmomatic) . Then, the clean reads were mapped to the reference genome sequence of the Sugar beet (GeneBank assembly accession, RefBeet-1.2.2) using the software "Hisat2" (version 2.2.1; http://daehwankimlab.github.io/hisat2/download/) with the default parameters. Sorting of reads was done using the software "Samtools" (www.htslib.org/download/), and subsequently the assembly of reads was performed using the software "StringTie" (https://ccb.jhu.edu/software/stringtie/). The software "Cuffdiff" (http://cole-trapnell-lab.github.io/cufflinks/) was used to identify genes, in which they were differentially expressed between every pair of samples. The q-value were considered as differentially expressed genes when gene expression is less than 0.05, and also their expression as an up-regulated or down-regulated was determined by the factor $\log_2$ fold change (FC), $+2 \leq \log_2 FC \leq -2$, respectively. We performed the enrichment analysis to evaluate the function and biological pathways of the differentially expressed genes (DEGs) by the software "KOBAS" (http://kobas.cbi.pku.edu.cn/home.do) using databases like the gene ontology (GO), encyclopedia of Kyoto genes, and genomes (KEGG). DEGs were significantly enriched in metabolic pathways when their Benjamini and Hochberg's-corrected *P*-values are less than 0.05. The volcano plot, heatmap, and Venn diagrams of the GO analysis were produced using the R package ggplots (https://www.rstudio.com).

Results

Assessment of RNA-seq data and mapping

The Illumina cDNA sequencing was conducted on libraries prepared from roots of two transgenic events, as well as two non-transgenic counterparts (WT). The RNA sequencing results of sugar beet roots were calculated through about 20.8 GB of data consisting of between 41 and 50 million of 150-bp paired-end raw reads for each library with Q20 above 97% and Q30 above 92%. These results indicate that the quality of RNA-seq data is adequate for the subsequent analysis. After trimming the reads, clean reads were mapped to the sugar beet reference genome (Accession No. RefBeet-1.2.2, Table 1).

Table 1. Statistics of the RNA sequencing results of sugar beet roots infested by the BNYVV.

Sample	Raw reads	Clean reads	Alignment rates (%)	Q20[a](%)	Q30[b](%)	GC(%)
WT1	43527574	43094660	69.34	97.47	92.69	43.00
WT2	45655544	45175322	68.54	97.44	92.65	42.64
S31	48711000	48154782	87.04	97.32	92.47	43.28
S33	41409562	40832888	66.46	97.47	92.97	43.26

[a,b]Q_{phred}, the base quality values; Q_{phred}=-10$\log_{10}$(e); Q20, error rate 1/100; Q30, error rate 1/1000.

Analysis of differentially expressed genes

The software "Cufflinks" was utilized to detect DEGs using a q-value<0.05 for reporting up- and down-regulated genes between S3 and WT. Overall, the expression of 37447 genes are compared between S3 and WT, out of which 343 genes were found as DEGs. This result corresponds to the difference in expression of about 0.9% of genes (310 upregulated; 33 downregulated) (Supplementary data file 1). In the present study, we focused on the most important genes and further narrowed down the number of DEGs. It was done by applying a stringent threshold for the log2-fold change or higher, so that it resulted in 308 DEGs within the S3_WT DEGs dataset (277 upregulated; 31 downregulated).

To illustrate the difference between plants, a heat map was provided using the R software for high differentially expressed genes in the S3 event versus WT (Figure 1). A volcano plot is also constructed using data shown in Figure 2. The volcano plot illustrates the relationship between FC and the statistical significance of DEGs.

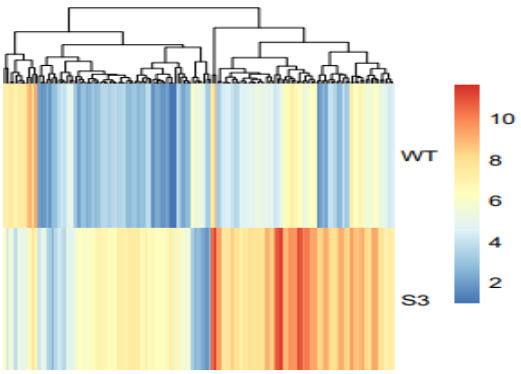

Figure 1. Heatmap generated for the differentially expressed genes of sugar beet roots infested by the BNYVV, which is reported by cufflinks analyses. Genes are arranged in descending order based on FC. Red and blue colors, respectively, represent higher and lower levels of gene expression.

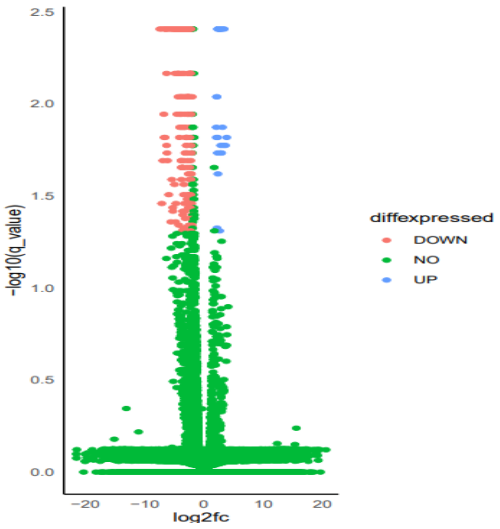

Figure 2. A volcano plot for the differentially expressed genes of sugar beet roots infested by the BNYVV. Each plotted dot indicates an individual gene. Green dots represent genes with no significant differentially expressed, whereas red and blue dots represent significant DEGs.

GO annotation and enrichment analysis of differentially expressed genes

In order to elucidate the possible changes in biological pathways, we performed a functional enrichment analysis of the DEGs based on the KEGG data using the software "KOBAS" (version 3.0). As shown in Figure 3, most of the changes occurred in metabolic pathways when comparing the infected transgenic event and WT transcriptomes. The increased ratios led to a health of the transgenic event despite of the BNYVV infestation.

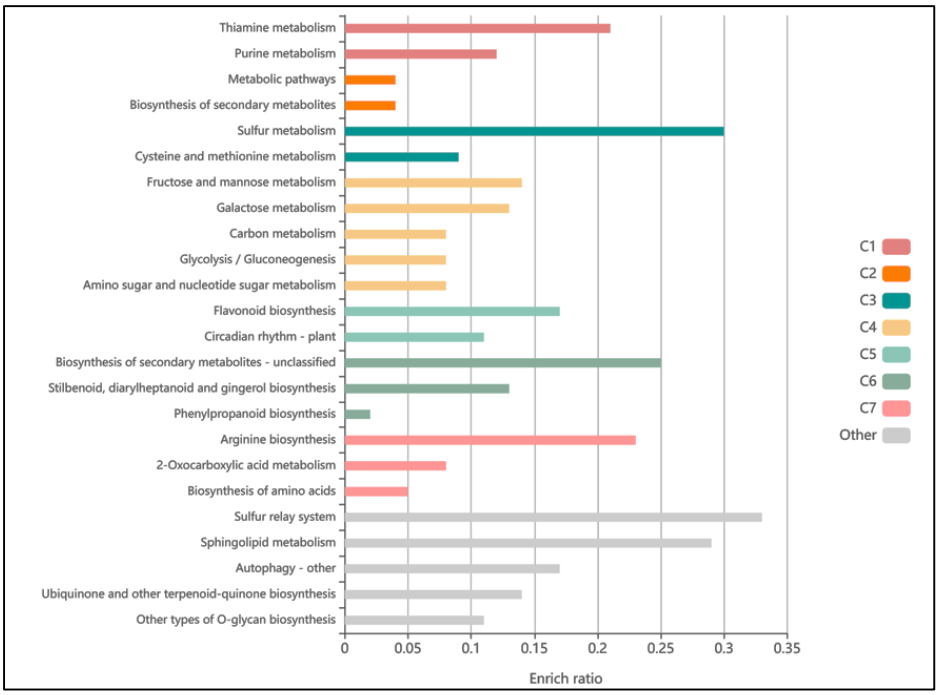

Figure 3. A bar plot showing the KEGG pathway enrichment of DEGs in S3_WT dataset for sugar beets infested by the BNYVV. S3_WT are up-regulated. In this bar plot, each row indicates an enriched function, and the bar length represents the enrichment ratio. The enrichment ratio is calculated as the "number of input genes"/"number of background genes".

The GO classification resulted in 66 terms for the identified genes with increased expression levels within the S3_WT dataset comparison. Overexpressions are considerable in 19 terms and 4 main biochemical pathways. These four major pathways are metabolism, environmental signal processing, genetic information processing and cellular processes. No pathway was found for genes with the reduced expression levels within the S3_WT dataset (Figure 3 and Supplementary data file 2).

According to analyses of the GO and KEGG pathway, 11 pathways including cellular processes, environmental information processing, genetic information processing, energy metabolism, organismal systems, lipid metabolism, amino acid metabolism, carbohydrate metabolism, metabolism of cofactors and vitamins, biosynthesis of secondary metabolites and nucleotide metabolism have shown significant enrichment ratios (Table 2). Such pathways are calculated as the "number of input genes"/"number of background genes". In a comparison between the transgenic S3 event and WT, the enriched GO terms were shown in 19 terms including sulfur relay system (3 out of 9 genes , 33.33 %), sulfur metabolism (7 out of 23 genes, 30.43%), sphingolipid metabolism (6 out of 21 genes, 28.57 %), biosynthesis of secondary metabolites – unclassified (3 out of 12 genes , 25 %), arginine biosynthesis (7 out of 31 genes , 22.58 %), thiamine metabolism (5 out of 24 genes , 20.83 %), flavonoid biosynthesis (9 out of 52 genes , 17.3 %), autophagy – other (5 out of 29 genes , 17.24 %), ubiquinone and other terpenoid-quinone biosynthesis (5 out of 36 genes , 13.88 %), fructose and mannose metabolism (7 out of 51 genes , 13.72 %), galactose metabolism (6 out of 48 genes , 12.5 %), purine metabolism (9 out of 77 genes , 11.68 %), cysteine and methionine metabolism (7 out of 79 genes , 8.86 %), carbon metabolism (17 out of 207 genes , 8.21 %), plant hormone signal transduction (15 out of 187 genes , 8.02 %), glycolysis / gluconeogenesis (8 out of 104 genes , 7.69 %), amino sugar and nucleotide sugar metabolism (8 out of 105 genes , 7.61 %), metabolic pathways (85 out of 1940 genes , 4.38 %) and biosynthesis of secondary metabolites (41 out of 974 genes , 4.20 %) were differentially expressed, so that all terms have been described as being up-regulated (Figure 3 and Supplementary data file 2). In summary, the

transgenic plants (S3) have revealed by up-regulation in some genes involved in the main pathways related to secondary metabolites, cellular processes, lipid metabolism, carbohydrate metabolism and amino acid metabolism (Table 2). Therefore, they are well-known defense responses of plants under a variety of stress conditions. As expected, plant hormone signal transduction, which is responsible for the plant-pathogen interactions, was also significantly up- regulated for the S3.

Discussion

Engineering of the virus-resistant transgenic crops by RNA silencing uses naturally defense mechanism of plants against viruses (Giudice et al., 2021). Moreover, a rapid cleavage of RNA transcripts up to the undetectable level and also lack of protein production reduce the safety risks related to toxic and allergenic compounds. These benefits make it a promising, sustainable, and environmentally friendly tool for the commercial release of virus-resistant transgenic products (Zare et al., 2015).

However, the main concern for transgenic crops is the public acceptance of this technology. Utilizing of exogenous DNA sequences into the plant genome may cause adverse effects such as silencing and/or modification of active genes, physical disruption, activation of silent genes, inactivation of endogenous genes, regulation of other genes through influencing biochemical pathways, and fusion protein formation (Jiang et al., 2017). The process of obtaining transgenic plants may affect the host plant genome. These concerns promoted the use of the unbiased high-throughput "omics" technologies to validate the substantial equivalence.

In the present study, we utilized the RNA-seq technique to investigate the possibility of detecting differentially expressed genes, which are derived from T-DNA insertion in the root of a transgenic sugar beet plants, S3-219 event. Our results show a differential expression of about 0.9% in the analyzed transcripts between S3 and WT.

Several studies used the RNA-seq technique to compare transcriptomes of transgenic plants with their non-transgenic counterpart to find possible changes in metabolism of papaya (*Coat protein of papaya ring spot virus* (PRSV) for resistance against the virus), wheat (*Glycine max* drought-responsive element-binding factor (GmDREB1) for drought- and salt-tolerant), *Bt* rice and maize (EPSPS and *Cry*1Ab genes) (Fu et al., 2019; Fu et al., 2021)(Fang et al., 2016; Jiang et al., 2017. Furthermore, several previous works that used microarray to investigate the transcriptome profile have reported similar results for wheat plants producing high level of gluten subunits (Baudo et al., 2006), rice plants that produce CsFv antibodies (Batista et al., 2008), glyphosate-resistant soybeans (Cheng et al., 2008), MON810 (insect-resistant maize), in which very limited transcriptional and diversity-dependent transcriptional regulations (Coll et al., 2008), this event, as well as glyphosate-tolerant NK603 maize, had fewer transcriptional differences than changes resulted from conventional breeding and environmental factors (Barros et al., 2010), rice plants that produce antifungal proteins (Montero et al., 2011) and barley plants that produce endo-chitinase involved in defense against stresses (Kogel et al., 2010).

However, a few studies have reported significant differences between transcripts of transgenic plants and their isogenic counterparts (Ben Ali et al., 2020; Ko et al., 2018; Lambirth et al., 2015). Schnell et al. (2015) indicated that the effects of insertion are inevitable results of genetic engineering, however, the introduction of unwanted traits is not. They also showed that genetic changes, such as the movement of transposable elements, the non-homologous end-binding process applied on double-stranded fractures and the intracellular transfer of organelle DNA, are comparable to the effects of insertion occurrence in plants. Thus, the effects of genetic engineering-related insertions are comparable to the genetic changes in naturally grown plants too. In light of this conclusion, a more extensive study of how genetic changes occur indicates plant genomes are constantly changing and, therefore, the effects of insertion have a relatively small contribution to the final genetic composition of plant species (Schnell et al., 2015).

The GO annotation and enrichment analysis of differently expressed genes exhibited the S3 event is shows by more changes in biological pathways than its non-transgenic counterparts. Some genes were all up-regulated in 11 biological pathways (Table 2) that can be due to resistance to the viral propagation.

Given that the study samples were taken from plants challenged with the virus, the difference in gene expression is not far-fetched. As a result of viral infections, plants can show changes in several metabolic pathways (Weiland et al, 2020). Carbohydrate metabolism changes in response to viral infections, which can be seen as changes in glucose levels and the expression of genes involved in glucose metabolism. Plants can also react to viral infections by altering the production of amino acids

which are required for plant defense responses and the multiplication of viruses (Kogovšek et al., 2016). When plants are exposed to pathogens, they produce secondary metabolites and pathogen-related proteins which are involved in plant defense (Kogovšek et al., 2016). It has also been suggested that differences between transgenic and non-transgenic materials may be observed under stress conditions. These findings indicate that the environment play an effective role on gene expression than gene modification as shown by previous publications, for instance in maize (Barros et al., 2010; Fu et al., 2021), rice (Batista et al., 2008; Fu et al., 2019), wheat (Baudo et al., 2006; Jiang et al., 2017), barley (Kogel et al., 2010), papaya (Fang et al., 2016) and soybean (Cheng et al., 2008). In addition, some studies have shown that inserting transgenes is very similar to the process of stress exposure, which triggers the expression of defense genes for adaptation and increased stress tolerance of transgenic plants (Fu et al., 2019; Montero et al., 2011; Jiang et al., 2017).

In the present study, patterns of variable expression of genes involved in biological pathways including lipids, amino acids, and carbohydrates metabolisms, cellular processes, biosynthesis of secondary metabolites and plant hormone signal transduction in S3 reflect significant gene expression changes, which occurred after gene insertion and may has an important role in plant resistance to the virus. In a previous study, it was also showed that alterations in the root protein profiles of transgenic plants were less than 8% compared to their non-transgenic counterparts. Different proteins are most likely related to the metabolism and defense/stress response (Hejri et al., 2021).

Conclusion

In this study, transcriptome analysis of silencing-induced transgenic sugar beet plants and non-transgenic counterparts were conducted while being exposed to the BNYVV. Our results show that there are slight differences in transcript, so that DEGs at significant levels consist of just 0.9% of transcriptomes of the parental wild-type plants versus the transgenic event. A number of DEGs are characterized as up-regulated genes, which is mainly related to pathogen resistance and stress tolerance, involved in carbohydrate, lipid, and amino acid metabolism, cellular processes, biosynthesis of secondary metabolites and hormone signaling pathways. These findings show that the T-DNA insertion does not activate unintended adverse effects following to gene expression in the roots of transgenic sugar beet. Many differentially expressed genes can be due to the enhanced pathogen resistance of transgenic sugar beet and well-being status of it. In our future study, we will survey the content of small-RNA produced through RNA silencing technique in transgenic plants. We will also investigate the presence of other viruses in the field-grown sugar beet plants which affects the severity of rhizomania disease.

Table 2. Significantly enriched KEGG pathways of sugar beet roots S3 _ WT infested by the BNYVV.

Main Pathway[a]	Pathway category	Enrich ratio
Environmental Information Processing	plant hormone signal transduction	8.02 %
Cellular Processes	Autophagy – other	17.24 %
Genetic Information Processing	Sulfur relay system	33.33 %
Organismal Systems	Circadian rhythm - plant	10.52 %
Energy metabolism	Sulfur metabolism	30.43 %
Lipid metabolism	Sphingolipid metabolism	28.57 %
Amino acid metabolism	Arginine biosynthesis; Cysteine and methionine metabolism	22.58 % 8.86 %
Metabolism of cofactors and vitamins	Thiamine metabolism; Ubiquinone and other terpenoid-quinone biosynthesis	20.83 % 13.88 %
Carbohydrate metabolism	Fructose and mannose metabolism;	13.72 %

	Galactose metabolism; Glycolysis	12.5 %
	Gluconeogenesis; Amino sugar and nucleotide	7.69 %
	sugar metabolism	7.61 %
Nucleotide metabolism	Purine metabolism	11.68 %
Biosynthesis of secondary metabolites	Flavonoid biosynthesis	17.30 %

[a] Only pathways with a p value of <0.05 were considered significantly enriched in this table.

Supplementary data

Supplementary data file 1. DEGs S3_WT dataset (XLS).

Supplementary data file 2. Function enrichment S3_WT up-regulated (XLS).

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Conflict of Interest

No conflict of interest was reported by the author(s).

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