A comparative morphological and transcriptomic study on autotetraploid *Stevia rebaudiana* (bertoni) and its diploid

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*Stevia rebaudiana* is an important medical plant for producing steviol glycosides (SGs) or stevioside. Autotetraploids (4x = 44) show an increasing level of morphology, physiology and tolerances comparing to diploids (2x = 22). However, little information regarded on the comparative transcriptome analysis between diploid and autotetraploid *S. rebaudiana* was found. In this study, synthetic autotetraploid was induced and nine SG compounds have been identified, synthesized in plastidal methyl erythritol 4-phosphate (MEP) pathway (Brandle and Telmer, 2007). According to previous reports, fifteen enzymes (DXS, DXR, CMS, CMK, MCS, HDS, HDR, GGDPS, CPPS1, KS1-1, KO1, KAH, UGT85C2, UGT74G1, UGT76G1) are mainly involved in steviol biosynthesis, in which kaurenoic acids (KAs) are converted to steviols rather than gibberellic acids (GAs) by KAH to form steviol glycosides (SGs) and downstream steviols are catalyzed by UGTs to directly produce steviosides and RAs (Yadav et al., 2011; Singh et al., 2017; Yang et al., 2015). Though it is still not very clear whether all SG compounds share the same biosynthesis route, stevioside and RA are known to be successively synthesized in plastidal methyl erythritol 4-phosphate (MEP) pathway (Brandle and Telmer, 2007). According to previous reports, fifteen enzymes (DXS, DXR, CMS, CMK, MCS, HDS, HDR, GGDPS, CPPS1, KS1-1, KO1, KAH, UGT85C2, UGT74G1, UGT76G1) are mainly involved in steviol biosynthesis, in which kaurenoic acids (KAs) are converted to steviosides rather than gibberellic acids (GAs) by KAH to form the 'backbone' of SGs and downstream steviols are catalyzed by UGTs to directly produce steviosides and RAs (Yadav et al., 2011; Singh et al., 2017; Yang et al., 2015).

Polyploidy plays a major role in the evolution history of plants by

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driving diversifications of morphology, physiology, and reproduction (Renny-Byfield and Wendel, 2014; Martin and Husband, 2013). Polyploidy plants generally developed with higher tolerances and duplicated chromosome counting and genome DNA content comparing with its progenitors (Wu et al., 2013; He et al., 2016; Sakhanokho and Islam-Faridi, 2014; Zhou et al., 2015). Genetic variability derived from polyploidy had been identified. Transcriptomic analysis of polyploidy using microarray or RNA-sequencing (RNA-seq) is widely used to uncover genome-wide alterations in gene expressions and mechanisms. Transcriptome studies of autotetraploid *A. thaliana*, birch (*Betula platyphylla*), rice, and other plants had been reported (Zhang et al., 2014; Wu et al., 2014; Mu et al., 2012; Yu et al., 2010; Jaskani et al., 2005). The significant alterations of polyploidy related genes and metabolism processes or pathways were identified, including genes related to energy metabolism and epigenetic regulation (such as DNA methylation), biosynthesis and signal transduction (e.g. indoleacetate (IAA) and ethylene) (Mu et al., 2012; Yu et al., 2010; Wang et al., 2004). Zhang et al. showed transcriptome alterations in *A. thaliana* autotetraploids was stable and developmentally specific (Yu et al., 2010).

Using comparative transcriptome analysis, researches showed that there were relative narrow alterations of gene expression in autopolyploids (Zhou et al., 2015). Weak differences in gene transcripts had found between diploid and autopolyploid potato (Stupar et al., 2007), birch (*Betula platyphylla*) (Mu et al., 2012), and Chinese woad (*Isatis indigotica* Fort.) (Zhou et al., 2015). Most of the differentially expressed genes or transcripts between diploids and autopolyploids were associated with biosynthesis or metabolism of secondary metabolites (Zhou et al., 2015; Madani et al., 2015). However, little information regarded on the comparative transcriptome analysis between diploid (2x = 22) and synthetic autotetraploid (4x = 44) *S. rebaudiana* was found till now.

In this study, we characterized the differences in morphological characteristics and content of SGs between autotetraploid and diploid *S. rebaudiana*. To investigate the differences in gene expression and the potential mechanism affect content of SGs with different ploidy, we performed the transcriptome analysis on both diploid and autotetraploid *S. rebaudiana* and carried out a comprehensive analysis of genetic changes. And we also demonstrated some plant resistant genes involved in ploidy change. These results would give us some new insights for understanding the phenotypic and physiological changes of autotetraploid *S. rebaudiana*, and the molecular mechanism related to increased SG biosynthesis in autotetraploid *S. rebaudiana*.

2. Results

2.1. Identification of artificial autotetraploid *S. rebaudiana* plants

After 0.20% colchicine treatment for 12 h, a total of 32.14% autotetraploid (2n = 4x = 44) plantlets were got from diploid donor adventitious buds (2n = 2x = 22). Cytological anatomy analysis showed autotetraploid *S. rebaudiana* plantlets doubled chromosome number (2n = 4x = 44, Fig. 1A), and flow cytometry analysis revealed the doubled DNA content in supposed autotetraploid plantlets (Fig. 1B). Autotetraploid *S. rebaudiana* plants showed larger leaves (Fig. 1C).

2.2. Polyploidy increases SGs contents

Fig. 2 shows the content of RA, rebaudioside B (RB) and total SGs in autotetraploid *S. rebaudiana* plants was higher than diploid plants (p < 0.05), while stevioside was lower than diploid plants (p < 0.05, Fig. 2). This suggested that polyploidy promoted not only the production of RA contents, but also the sweetness quality of SGs in *S. rebaudiana* plants.

![Fig. 1. Ploidy analysis and morphological characterization of autotetraploid *S. rebaudiana* plants.](image1.png)

![Fig. 2. Content of steviol glycosides in leaves of autotetraploid and diploid (4x = 44) *S. rebaudiana* plants.](image2.png)
annotation, these genes were assigned to 58 GO categories at the second level (Fig. S2) and 46 KEGG categories (Fig. S3). Cellular process (31,849 genes, 31.59%), Metabolic process (22,284 genes, 22.78%), Carbohydrate metabolism (3184 genes, 6.38%) pathways.

2.4. Identification, clustering, and enrichment analysis of differentially expressed genes (DEGs) analysis

To reveal the differences caused by ploidy in *S. rebaudiana*, we performed a comparative analysis with six leaf transcriptomes. With the thresholds of fold change ≥ 1.5 and FDR ≤ 0.05, we identified 4114 DEGs (2.8%), including 2105 up-regulated DEGs (51.17%) and 2009 down-regulated DEGs (48.82%) in autotetraploids leaf compared with diploids (Fig. 3A). The gene list is shown in Table S1. Clustering analysis obviously sorted these six samples into two groups according to the expression levels of DEGs (Fig. 3B).

To uncover potential biological processes during ploidization, GO term enrichment analysis was conducted to query the significantly altered genes in autotetraploid *S. rebaudiana* leaves. It was found that GO biological processes of “secondary metabolite biosynthetic process”, “secondary metabolic process”, “triglyceride biosynthetic process” and “pectin catabolic process” were significantly enriched (q value < 0.05) in up-regulated DEGs, while down-regulated DEGs were significantly involved with biological processes of “secondary metabolite biosynthetic process”, “flavonoid glucuronidation”, “flavonoid biosynthetic process” and “oxylipin biosynthetic process” (Table S2). Additionally, significantly enriched (correct p-value ≤ 0.05) pathways based on KEGG enrichment analysis were detected in up and down-regulated DEGs (Fig. 4). Stratified up-regulated DEGs were related with “Diterpenoid biosynthesis” [7 genes, including up-regulated UDPGT, KSL protein, CYP91A1, GA3ox1, GA2ox1 and GA2ox-encoding genes], “Phenylpropanoid biosynthesis” [16 genes, including GH-catalytic, CATL, PAL, UDPGT, probable At4g26220 (CCoAOMT) isoform X1 and AD1-encoding genes], “Flavonoid biosynthesis” [5 genes, including probable CCoAOMT, CATL and CHS-encoding gene], “Cutin, suberine and wax biosynthesis” [5 genes, including FAH, CYP450, CATL and FAGoAR1-encoding gene], “Starch and sucrose metabolism” [12 genes, including GH-catalytic, CATL, PAL, UDPGT, probable At4g26220 (CCoAOMT) isoform X1 and AD1-encoding genes], “Stilbenoid, diarylheptanoid and flavonoid glucuronidation” [4 genes, including GST, CYP91A1, probable CCoAOMT and CATL-encoding genes], “Stilbenoid, diarylheptanoid and flavonoid glucuronidation” [4 genes, including GST, CYP91A1, probable CCoAOMT and CATL-encoding genes]. The top 10 KEGG pathways, including 4114 genes and CKX, were significantly altered (p value ≤ 0.05) pathways, are listed in Table S3.

Down-regulated DEGs in autotetraploid *S. rebaudiana* leaves were associate with “Flavone and flavonol biosynthesis” [6 genes encoding UDPGT encoding genes and F3′H], “Linoleic acid metabolism” [7 genes encoding PLAT2], “Zeaxin biosynthesis” [6 genes encoding UDPGT genes and CKX], “Glutathione metabolism” [10 genes encoding GST, RDRLL protein and GPX], “Stilbenoid, diarylheptanoid and flavonoid glucuronidation” [4 genes encoding PLOA/SOA; Table S3].
2.5. Analysis of genes related to SGs biosynthesis

It has been reported that at least 15 genes involved in the SG biosynthesis in *S. rebaudiana* (Brandle and Telmer, 2007). In our study, it was found that 10/15 (66.6%) genes were up-regulated in autotetraploids leaves with a range of 15%–100% compared with diploids, higher than up-regulated ratio (51.17%) in DEGs (Fig. 5A and B). Among those, 2 of 15 genes, CPPS1 and KAH, were identified to be up-regulated differentially expressed genes (P-value < 0.5), probably to be key enzymes in SGs biosynthesis, which were in agree with previous reports (Jin et al., 2018; Bondarev et al., 2001). Further qRT-PCR analysis showed similar expression patterns as found in NGS data, where CPPS1 and the heatmap clustering of DEGs in samples from autotetraploid and diploid seemed to be activated in the autotetraploid (Fig. 6). These results suggested that SGs biosynthesis pathway was got subsequently. Overall, 32,533 genes with SGs biosynthesis, WGCNA was performed and modules associated with SGs biosynthesis, DNA replication and nucleosome assembly, processes including protein ubiquitination and catabolic activity, phenylalanine, tyrosine and tryptophan biosynthesis, plant morphogenesis, plant growth, and plant MAPK signaling pathway were enriched in "Photosynthesis" [4 genes encoding Photosystem I Psaf, reaction centre subunit III and meiosis specific protein SPO22], "Fructose and mannose metabolism" [3 genes encoding protein PNS1, Hexapep domain-containing protein/NTP transferase domain-containing protein and PFK-2/FBPase-2 isofom X3], "D-Glutamate and D-glutamate metabolism" [GDH1 encoding gene], “plant MAPK signaling pathway” [SAPK1-like and PR-1 encoding genes] and “Phenylpropanoid biosynthesis” (UGDPT and ALDH2C4 encoding genes), whereas the down-regulated genes were enriched in “Tropaeane, piperidine and pyridine alkaloid biosynthesis” [2 genes encoding class I/classII aminotransferase and cytoplasmic AST], “Isoquinoline alkaloid biosynthesis” [genes encoding cytoplasmic AST], “Porphyrin and chlorophyll metabolism” [genes encoding HAS or Cox15 and Chlorophyllase], alpha-Linolenic acid metabolism [PLAT/LH2 and JMT encoding genes] and “Phenylalanine, tyrosine and tryptophan biosynthesis” (AST encoding genes, Table S5). These results indicated that enhanced resistance in autotetraploid *S. rebaudiana* was related with altered expression of genes related to secondary metabolism.

2.6. Identification of DEGs related to resistance in autotetraploid *S. rebaudiana*

Since autotetraploidy increase plant resistance to abiotic stresses, including drought, salt and heat (Zhu and Liu, 2018; Yu et al., 2016; Schumann et al., 2017), we next implemented the identification of genes related to the resistance in autotetraploid *S. rebaudiana*. The PlantPreS database was introduced and the overlapped or genes with high identity in our study were identified. Overall, 3679 genes, including 296 DEGs (152 up-regulated and 144 down-regulated DEGs), were identified. Additionally, 11 resistance genes were selected to validate using qRT-PCR, showing a consistent up-regulation in autotetraploids when compared to NGS results (Fig. 8). Further KEGG pathway analysis showed these up-regulated genes were involved in “Photosynthesis” [4 genes encoding Photosystem I Psaf, reaction centre subunit III and meiosis specific protein SPO22], “Fructose and mannose metabolism” [3 genes encoding protein PNS1, Hexapep domain-containing protein/NTP transferase domain-containing protein and PFK-2/FBPase-2 isofom X3], “D-Glutamate and D-glutamate metabolism” [GDH1 encoding gene], “plant MAPK signaling pathway” [SAPK1-like and PR-1 encoding genes] and “Phenylpropanoid biosynthesis” (UGDPT and ALDH2C4 encoding genes), whereas the down-regulated genes were enriched in “Tropaeane, piperidine and pyridine alkaloid biosynthesis” [2 genes encoding class I/classII aminotransferase and cytoplasmic AST], “Isoquinoline alkaloid biosynthesis” [genes encoding cytoplasmic AST], “Porphyrin and chlorophyll metabolism” [genes encoding HAS or Cox15 and Chlorophyllase], alpha-Linolenic acid metabolism [PLAT/LH2 and JMT encoding genes] and “Phenylalanine, tyrosine and tryptophan biosynthesis” (AST encoding genes, Table S5). These results indicated that enhanced resistance in autotetraploid *S. rebaudiana* was related with altered expression of genes related to secondary metabolism.

3. Discussion

3.1. Ploidy induction in stevia

Polyploidy shows enlarged organs, prolonged vegetative growth, bigger vegetative or reproductive organs and higher stresses tolerance in comparison with its parents (Martin and Husband, 2013; Zhou et al., 2015; Jaskani et al., 2005; Madani et al., 2015; Liu et al., 2011; Lucia et al., 2015; Hollister et al., 2012). We successfully produced autotetraploid (4x) *S. rebaudiana* using colchicine treatment with doubled chromosome number and DNA content. Furthermore, we confirmed the autotetraploid *S. rebaudiana* plant on its morphological and
physiological characteristics, which showing larger leaves and higher SGs contents. Also, it was reported that, among the components of SGs, the ratios of stevioside and RA were altered during different development stages, higher RA than stevioside from vegetative to flower stage (Yang et al., 2015; Bondarev et al., 2003). And it was measured that the contents of rebaudioside A and B in autotetraploids were higher than diploids but stevioside content showed less, implying an improved transformation from stevioside to RA and a promotion for organoleptic quality and sweetness after ploidy induction.

3.2. RNA-seq on stevia

To investigate potential molecular change during ploidization, we conducted a transcriptome comparison between 4x *S. rebaudiana* and diploid using RNA-seq. We constructed leaf, stem and root libraries to assemble a more reliable reference transcript. Given leaves served as the main organ for both synthesis and primary accumulation of SGs (Bondarev et al., 2003), we focused on differential expression genes in leaf and only 4114 DEGs (2.8%: 1.43% up-regulated, 1.37% down-regulated) were detected by paired comparison. And it was referred that much gene silencing and activation occur in allopolyploid plants but may not occur in the process of autopolyploidization (Lu et al., 2006; Pignatta et al., 2010). In agreement with reported result of *Chrysanthemum lavandulifolium* (Fisch. ex Trautv.) Makino (Gao et al., 2016), only a small number of DEGs were found between autotetraploids stevia and its parents, as well as up/down-regulated DEGs ratios.

3.3. Genes related to SGs biosynthesis

In our analysis, 2 of 15 SGs biosynthesis genes, CPPS1 and KAH were considered as significant differential expressed genes (P-value < 0.05). Interesting, there still exists 10/15 (67%) genes to be up-regulated in autotetraploids leaves by a range of 15%–100% compared with diploids, higher than up-regulated gene ratio in DEGs (51.17%), implying SGs biosynthesis pathway was activated during ploidization. CPPS1 and UGT74G1 were validated to be significantly up-regulated in autotetraploids leaves by RT-qPCR, partial consistent with NGS results. It was reported that the importance of CPPS1 in determining SGs content by preventing over-expression from interfering with normal GA
metabolism (Humphrey et al., 2006; Richman et al., 1999). Previous study by Mohamed et al. revealed that the rate-limiting step in the glycosylation pathway of SGs was the formation of steviolmonoside by glycosylation of steviol, which was controlled by UGT74G1, UGT76G1 and UGT85C2 (Mohamed et al., 2011; Behroozi et al., 2017). And transcription of UGT74G1 was shown to significantly decrease under dehydration treatment, indicating associated with environment response (Yang et al., 2015).

To further understand the molecular mechanism of increased SG contents in autotetraploids, we performed the WGCNA analysis to explore genes associated with SG biosynthesis. As expected, we found 7 modules (25,441 genes) were related to the SG biosynthesis, which genes were mostly up-regulated and associated with photosynthesis, plant morphogenesis, plant growth and other GO biological processes including secondary metabolic, flavonoid biosynthetic and glycine metabolic processes, indicating those genes were activated during ploidization, and promoted the SGs accumulation indirectly via various biological processes, in addition to directly regulation in MEP pathway.

3.4. Genes related to resistances

Autotetraploid plants showed superiorities in stresses tolerances, defense responses, quality, and environmental adaptation (Madani et al., 2015; Lucia et al., 2015; Hollister et al., 2012; Yang et al., 2014; Yan et al., 2015). Among DEGs induced by autotetraploidy in S. rebaudiana plants, there were 296 DEGs were related with plant resistance based on PlantPReS database. These genes, including up-regulated AST, PLAT2 and JMT encoding genes, were associated with "Tropane, piperidine and pyridine alkaloid biosynthesis", "Isoquinoline alkaloid biosynthesis", and "Phenylalanine, tyrosine and tryptophan biosynthesis". JMT is a key enzyme for JA regulated response in plant (Seo et al., 2001). The expression of JMT in pepper was induced by wounding and methyl jasmonate application (Song et al., 2005), while in strawberry JMT was negatively correlated with the fruit development (Preuß et al., 2014). PR-1, a gene encodes pathogenesis-related protein 1, was up-regulated in autotetraploid S. rebaudiana plants comparing with its diploid parents. PR-1 encodes an extracellular protein which is stimulated by pathogen challenge and involved in immune and acquired resistance in plants (Gamir et al., 2017; van Loon et al., 2006; Alexander et al., 1993). In addition, several genes encoding GELP
were up-regulated in autotetraploid *S. rebaudiana* plants. GELPs are a newly discovered lipolytic enzyme subfamily with limited knowledge of functional properties (Dong et al., 2016; Chepyshko et al., 2012). Overexpression of a salt-induced gene encoding a GDSL-motif lipase LTL1 increased salt tolerance in yeast and transgenic *Arabidopsis* plants (Naranjo et al., 2006). Dysregulation of EXL6 (extracellular lipase 6) in *Brassica rapa* ssp. *pekinensis* played a great role in pollen development (Dong et al., 2016). With the analysis of plant morphological
characteristics and functional enrichment of DEGs in autotetraploid *S. rebaudiana* plants, we supposed that those DEGs including plant GDSL enzymes play important roles in metabolisms and biosynthesis of secondary metabolites that accounting for the differences in autotetraploid *S. rebaudiana* plants to diploid plants. Taken together, these results in our study demonstrated the more complex resistance systems in autotetraploid *S. rebaudiana* plants than its diploid parents.

In conclude, we provided evidence that autotetraploidization can alter the SGs biosynthesis and resistant genes expression in stevia. This finding might advance further understanding of the complexity of morphological and molecular mechanisms on autotetraploids, which are of great relevance to cultivation and breeding of stevia.

4. Materials and methods

4.1. Plants materials

*Stevia rebaudiana* bertoni (*S. rebaudiana*, Shoutian 3, 2n = 22) was obtained from Jiangsu Germplasm Repository and used as diploid donor. Tender stem segments with two leaves and one internode were surface-sterilized (70% ethanol, 30 s 0.1% HgCl2, 8 min) and inoculated in MS medium (containing 0.3 mg/L 6-BA, 0.2 mg/L KT, and 0.05 mg/L NAA) for 20 days. Induced adventitious buds were prepared for autotetraploid induction.

4.2. Autotetraploid induction

Induced adventitious buds were subjected to 0.20% colchicine or sterile water (control) for 12 h, following with inoculation in MS medium for 30 days, at 24 ± 1 °C, with a light cycle of 12 D: 12L. Then, seedlings were transferred to 1/2 MS medium (80 mM KCl, 20 mM Na2EDTA, 1% (v/v) Triton X-100, 0.117% (v/v) β-mercaptoethanol, pH = 7.5). Samples were then filtered (50 μm nylon filter), and centrifuged. The nuclei suspensions were collected and stained with DAPI for 10 min. Ploidy level analysis using a Partec flow cytometric analysis as previously described using a Partec flow cytometer (Partec, Münster, Germany). Diploid *S. rebaudiana* was used as control.

4.3. Chromosome counting

Root tips (~5 mm in length) of diploid and autotetraploid seedlings were cut off and chromosome numbers were determined (He et al., 2016; Madani et al., 2015). In brief, root tips were treated with 8-hydroxyquinoline (C9H7NO) at 20 °C for 4 h, and fixed with Carnoy’s solution (glacial acetic acid: 95% ethanol, 1:3) at 4 °C for 24 h. Samples were then hydrolyzed using 1 mol/L HCl at 60 °C for 10 min. Chromosome counting was performed using 1 mm hydrolyzed root tips by soaking in a drop of Carbol fuchsin for 10 min and squashing on the microscopic slide. Chromosome numbers were counted under a light Olympus microscope (magnification × 100).

4.4. Flow cytometric analysis

DNA content of supposed diploids and autotetraploids were analyzed using flow cytometric analysis as previously described using leaves (He et al., 2016; Zhou et al., 2015). Leaves were cleaned, cut up, and incubated with cold isolation buffer [15 mM Tris, 20 mM NaCl, 80 mM KCl, 20 mM Na2EDTA, 1% (v/v) Triton X-100, 0.117% (v/v) β-mercaptoethanol, pH = 7.5]. Samples were then filtered (50 μm nylon filter), and centrifuged. The nuclei suspensions were collected and stained with DAPI for 10 min. Ploidy level analysis using a Partec flow cytometer (Partec, Münster, Germany). Diploid *S. rebaudiana* was used as control.

4.5. SGs measurement

The contents of RA, steviodise, RB, and total SGs were measured using high performance liquid chromatography (HPLC) methods. Dried leaf samples (5 g) from autotetraploid and diploid *S. rebaudiana* plants were extracted using water (100 mL) extraction methods, followed with purification (macroporous adsorbents, AB-8), elution (80% methanol), and filtration (0.45 μm). Two milliliters of the filtrate was loaded on the cartridge and allowed to flow through. The cartridge was washed with water followed by acetonitrile:water (78:22, v/v) and air dried for 3 min. Samples were then eluted in 1 mL of methanol:acetonitrile (50:50, v/v) and filtered using a 0.45 μm nylon centrifuge tube (Corning). HPLC analysis of the samples was carried out on a Shimadzu LC-10A HPLC using a Zorbax-NH2 column (250 mm × 4.6 mm; 5 μm) and detected by a photodiode array detector (SPD-10A with high-sensitivity cell). Five microliters of sample was injected, and the elution was performed over 24 min with a 30%–80% acetonitrile gradient at a flow rate of 1.0 mL min⁻¹ according to protocol by Shimadzu. Column oven was maintained at 40 °C. Peak assignment for the absorbance spectrum was based on comparison with elution profile of known standards (complete Stevia standards kit, KIT-00019565-005, ChromaDex) at a wavelength of 210 nm. HPLC analysis of Stevia samples was done at least in biological triplicates.
4.6. RNA extraction, library preparation, Illumina sequencing

Young leaves of diploid and autotetraploid adult plants were collected at 60 days post transplantation (at vegetable growing stage) in the fields at experimental station of Nanjing Agricultural University (118.85 °E, 32.04°N) at May 18 of 2017. Young and middle leaves were collected, snap-frozen in liquid nitrogen and stored at −80 °C for RNA sequencing before isolation of total RNA. Isolated total RNA samples were dealt with using RNase-free DNase I (Takara, Japan). Agilent 2100 Bioanalyzer (Agilent, CA, USA) and ABI StepOnePlus Real-Time PCR System were used for RNA quantification and qualification (Zhou et al., 2015). 18 sequencing libraries (from 6 plants) were prepared and applied for Illumina HiSeq sequencing (HiSeq 4000, San-Diego, CA, USA) in PE150 strategy.

4.7. Data processing and de novo assembly

Raw reads were got and were quality-filtered (low-quality, adaptor-polluted and high content of unknown base reads). De novo assembly with clean reads was performed using Trinity (version 2.0.6) (Grabherr et al., 2013). CD-Hit (version v4.6.4) (Fu et al., 2012) was used for clustering transcripts to genes at 90% sequences similarity. The remained contigs were considered as the reference sequences.

4.8. Functional annotation of assembled sequences

We used BLASTx (version 2.5.0, default parameters) (Camacho et al., 2009) to align genes to NR (non-redundant database), COG (Clusters of Orthologous Groups), Pfam, KEGG (Kyoto Encyclopedia of Genes and Genomes) and SwissProt to get the annotation. Blast2GO (Conesa et al., 2005) program (version 2.5.0, default parameters) was used for GO annotation of genes. KEGG pathway annotation was performed using KOBAS (Xie et al., 2011) software (v3.0) against the KOBAS database.

4.9. Identification of DEGs

The expression levels of all genes were normalized by FPKM (fragments per kilobase of exon per million reads mapped). DEGs in genes were detected using edgeR (Robinson et al., 2010) R package (version 3.10.5) and Fisher’s exact test, with the thresholds of fold change (FC) ≥ 1.5 and false discovery rate (FDR) ≤ 0.05. Hierarchical clustering for DEGs was performed with pheatmap, a function of R. For clustering more than two groups, we perform the intersection and union DEGs between them, respectively.

4.10. GO and pathway enrichment analysis of DEG

The clean reads of the diploid and autotetraploid samples were mapped back to de novo assembly results using RSEM (Li and Dewey, 2011). The GO enrichment of DEGs (Fold change ≥ 1.5 and FDR ≤ 0.05) was performed using clusterProfiler R package (version 3.6.0) (Yu et al., 2012). For KEGG pathway enrichment, KOBAS program was used. Terms with a correct p-value ≤ 0.05 were defined as significant enriched.

4.11. Identification of genes related to plant resistance

To obtain the changed profiles in autotetraploid plants, we searched the genes related to plant resistance by blasting the DEGs against PlantPReS database (www.proteome.ir). Items with less than e−10 (bi-directional best hit) were identified as DEGs associated with resistance in autotetraploid plants.

4.12. Mining of genes related to stevioside synthesis

Co-expression network was conducted using WGCNA (Langfelder and Horvath, 2008) (residuals weighted gene coexpression network analysis) R packages (version 1.61). Low-expression annotated genes of all 18 RNA-seq samples were previously filtered based on FPKM > 0.5 in at least six samples, resulting in 32,533 genes. The residuals were used for one-step network WGCNA construction. Module detection protocol was applied with a power function (beta = 8) to a pair-wise gene correlation (Pearson’s) matrix, initially estimated using per gene residual values. Modules containing genes related with SG synthesis were selected and were subjected to enrichment of GO “Biological Process” terms using clusterProfiler. Full set of genes input into WGCNA was used as background.

4.13. qRT-PCR

Total RNA was extracted from young leaves and prepared as previously described. QRT-PCR was performed using specific primers (Table S6) on an ABI PRISM 7500 real-time PCR System (Applied Biosystems, USA) in a volume of 10 μL [5 μL of 2 X SYBR Green MasterMix reagent (Thermo Fisher Scientific, Rockford, IL, USA), 1 μL of cDNA, and 0.2 μL of each primer (10 μM)] following the conditions as follows: 95 °C for 5 min; 40 cycles of 95 °C for 15 s and 60 °C for 45 s; a temperature-ramping step from 95 °C to 65 °C. All samples were examined in triplicates of three biology replicates and accompanied with negative controls. β-actin was used as the internal control. The mRNA relative expression of each gene was calculated using the 2−ΔΔCt method.

4.14. Statistical analysis

Data of SGs contents and genes’ relative expression level were expressed as mean ± SD. Differences between groups were analyzed using t-test in GraphPad Prism 6 (Graphpad Software, San Diego, CA, USA). p < 0.05 was regarded as significantly different.

5. Conclusions

In this study, we used the RNA-seq and bioinformatics analysis to obtain a comprehensive transcriptome of S. rebaudiana and mRNAs expression profiles among diploids and autotetraploids. We screen polyploidy-related mRNAs in leaf tissue by pairwise comparison. Applying functional enrichment analysis, we assumed that DEGs were associated with SGs biosynthesis, plant growth and secondary metabolism, which proved to be impact phenotypes in polyploidy compared to diploid. Moreover, WGCNA showed co-expressed genes of key SG biosynthesis pathway genes were enriched in functional categories including photosynthesis, flavonoid and secondary metabolic process, plant growth and morphogenesis.

Our findings has highlighted molecular changes related to SGs metabolism of polyploidy, and our results also provided information in understanding molecular mechanism involved plant resistance responsible for phenotypic change or physical traits of autotetraploid.

Conflicts of interest

The authors have declared that no competing interests exist.

Contributions

Zeng-xu Xiang conceived and designed the experiments. Experiments were performed by Xing-li Tang. Data analysis was performed by Wei-hu Liu. All authors participated into paper writing.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.003.

Abbreviations

SG
steviol glycoside
gEG
differentially expression gene
tT2D
type 2 diabetes mellitus
RA
rebaudioside A
RB
rebaudioside B
Mep1
methyl erythritol 4-phosphate
Dxs
1-deoxy-D-xylulose 5-phosphate synthase
Dxr
1-deoxy-D-xylulose 5-phosphate reductoisomerase
Cmk
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
Mcs
2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
Hds
(E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase
HDr
IPP/DHAP synthase
Ggdps
Geranyl geranyl diphosphate synthase
Cps1
copalyl pyrophosphate synthase
Ks1-1
Kaurene synthase
Ko1
eni-kaurene oxidase CYP701A5
Kah
eni-kaurenoic acid 13-hydroxylase
Ugt85C2
UDP glucosyltransferase – 85C2
Ugt74G1
UDP glucosyltransferase – 74G1
Ugt76G1
UDP glucosyltransferase – 76G1
UDPgt
UDP-glucuronosyl/UDP-glucosyltransferase
Ksl
kaurene synthase like protein
Ga3ox
giberellin 3-oxidase
Gh
glycoside hydrolase
gH-
catalytic glycoside hydrolase, catalytic domain-containing protein
cat
chalconemophilic acetyltransferase
CATL
chalconemophilic acetyltransferase-like domain-containing protein-like
Pal
phenylalanine ammonia-lyase
CcoAOMT
caffeoyl-Coa O-methyltransferase
Ad1
alcohol dehydrogenase 1
Chs
chalcone synthase
Fah
fatty acid hydrolase
CYP450
cytochrome P450
Facoar1
f预算-specific fatty acyl-Coa reductase 1
Gt
glycosyl transferase, family
ThrPj
trehalose phosphate phosphatase J
Cw13
cell wall invertase
Fl3h
flavonoid 3’-hydroxylase
Plat2
lipase/lipoxygenase, PLAT/LH2 family protein
Ckx
cytoxin oxidase
Gt
glutathione S-transferase
RdrL
ribonucleoside-diphosphate reductase large subunit-like
Gpx
glutathion peroxidase
PlOa/Soa
prenyltransferase/squalene oxidase
Pfk-2/FbPase-2
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
Gdh1
glutamate dehydrogenase 1
Sapk
serine/threonine-protein kinase
Pr-1
pathogenesis-related protein 1
Aldh2C4
aldehyde dehydrogenase family 2 member C4-like

AST
aspartate aminotransfere
HAS
Heme A synthase
Coxl5
cytochrome C Oxidase Assembly Homolog
Jmt
jasmonic acid carboxyl methyltransferase
Gelp
GDSL esterase/lipase

References


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