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Research article

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 morphological features were confirmed. A comprehensive transcriptome of stevia leaf, stem and root from the diploids and autotetraploids was constructed based on RNA-seq, yielded 1,000,892,422 raw reads and subsequently assembled into 251,455 transcripts, corresponded to 146,130 genes. Pairwise comparisons of the six leaf libraries between the diploids and autotetraploids revealed 4114 differentially expression genes (DEGs), in which 2105 (51.17%) were up-regulated in autotetraploids and associated with SGs biosynthesis, plant growth and secondary metabolism. Moreover, weighted gene co-expression network analysis showed co-expressed genes of fifteen genes of SG biosynthesis pathway were enriched in photosynthesis, flavonoid and secondary metabolic process, plant growth and morphogenesis. A hundred of DEGs related to plant resistance were identified by interviewing PlantPReS database. This study has highlighted molecular changes related to SGs metabolism of polyploidy, and advanced our understanding in plant resistance responsible for phenotypic change of autotetraploids.

1. Introduction

Stevia rebaudiana (*S. rebaudiana*) is an important medical plant that accumulates steviol glycosides (SGs) or stevioside. SGs are natural diterpenoid ingredients of non-toxic and non-caloric sweeteners that widely used as alternative to sugar in foods and beverages (Abbas Momtazi-Borojeni et al., 2017; Carakostas et al., 2008; Barriocanal et al., 2008). The administration of SGs shows safe and effective medicinal properties for diseases including cardiovascular diseases, diabetes, and hyperglycaemia (Carakostas et al., 2008; Onakpoya and Heneghan, 2015; Philippaert et al., 2017; Chatsudthipong and Muanprasat, 2009). With the worldwide increasing concerns on human health, the demand for safe and non-nutritive sweeteners is rising, especially in the population with type 2 diabetes mellitus (T2D), obesity, hypertensive, inflammatory and immune diseases, and people with diet conscious (Abbas Momtazi-Borojeni et al., 2017; Carakostas et al., 2008). These increase the market requirement for SGs, as well as the requirement for *S. rebaudiana* cultivation and breeding.

It is indicated that at least eight SG compounds have been identified in leaves of stevia, in which stevioside and rebaudioside A (RA) are the two main glycosides (Yadav et al., 2011). It is reported that stevioside and RA are the most two highest proportion of dry weight of leaves (5–10% and 2–4%) and the most two sweetest glycosides (about 200–300 times) relative to sucrose, meanwhile, the ratio of RA to stevioside is an accepted measure to evaluate sweetness quality (Yadav et al., 2011; Brandle and Telmer, 2007; Purkayastha et al., 2016). 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 steviols 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.

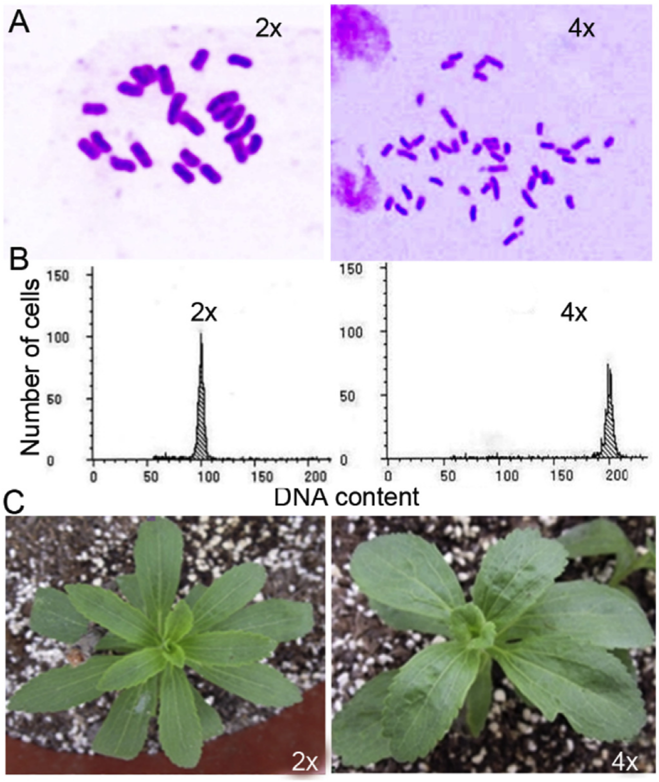

Fig. 1. Ploidy analysis and morphological characterization of autotetraploid *S. rebaudiana* plants. A, chromosome counting at mitotic metaphase of 2x (2n = 22) and 4x (2n = 44) *S. rebaudiana* plants. Magnification × 100. B, flow cytometric analysis of DNA content. C, seedlings of 2x (2n = 22) and 4x (2n = 44) *S. rebaudiana* plants.

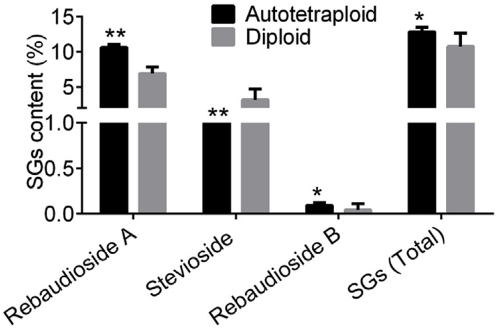

Fig. 2. Content of steviol glycosides in leaves of autotetraploid and diploid (4x = 44) *S. rebaudiana* plants.

2.3. Overview of RNA-seq, de novo assembly, and annotation

We obtained a total of more than 1000 million raw reads from 18 cDNA libraries, which generated 148.67 Gb clean bases after quality control (Table 1). The high-quality reads were available in the NCBI SRA database (accession number: SRP217359). All clean reads were assembled to 251,445 transcripts, corresponding to 146,130 genes. The average length, N50 and GC content of all transcripts was 847.78bp, 1385bp, and 43.82%, respectively (Table 2). Genes accounting for 52,343 (NR, 35.82%), 42,396 (Swissprot, 29.01%), 49,877 (KEGG, 34.13%), 35,931 (KOG, 24.59%), 22,252 (Pfam, 15.23%), and 38,365 (GO, 26.25%) in six functional databases, respectively (Table 3). As regards to species distribution, 13.25% distinct sequences of the NR annotated genes top matched to sequences from *Cynara cardunculus* var. *scolymus* (53.01%), *Daucus carota* subsp. *sativus* (2.73%), *Vitis vinifera* (2.28%) and *Helianthus annuus* (1.5%, Fig. S1). With the GO and KEGG

Table 1
Summary of sequencing reads after filtering.

Sample	tissue	libraries	RawReads	CleanReads	CleanBase(Gb)	Q30(%)	Ratio(%)
Diploid	leaf	DL-1	49622190	49144808	7.37	94.81	99.04
		DL-2	49670858	49198440	7.38	94.39	99.05
		DL-3	52515490	52064358	7.81	94.84	99.14
	root	DR-1	55126698	54502474	8.18	93.93	98.87
		DR-2	58879076	58254686	8.74	94.01	98.94
		DR-3	56828576	56286754	8.44	94.36	99.05
	stem	DS-1	54154450	53620350	8.04	94.74	99.01
		DS-2	62241572	61648778	9.25	94.66	99.05
		DS-3	62690978	62087476	9.31	94.55	99.04
Autotetraploid	leaf	TL-1	53840174	53350144	8	94.66	99.09
		TL-2	53534186	53021284	7.95	94.57	99.04
		TL-3	54177310	53694458	8.05	94.6	99.11
	root	TR-1	57691660	57089692	8.56	94.08	98.96
		TR-2	57476008	56919142	8.54	93.98	99.03
		TR-3	46931816	46420772	6.96	94.41	98.91
	stem	TS-1	57607366	57075018	8.56	94.81	99.08
		TS-2	62301380	61731866	9.26	94.75	99.09
		TS-3	55602634	55104064	8.27	94.54	99.10

Q30: the rate of bases which quality is greater than 30.
Ratio: the rate of cleanreads to rawreads.

Table 2
Quality metrics of transcripts.

Item	Value
Total number of sequences	251445
Total number of genes	146130
Total base of sequences (Mb)	213
Maximum sequence length (bp)	16820
Minimum sequence length (bp)	200
Average sequence length (bp)	847.78
Median contig length (bp)	511
N50 (bp)	1385
Percent GC (%)	37.21

N50: a weighted median statistic that 50% of the TotalLength is contained in Unigenes great than or equal to this value. GC (%): the percentage of G and C bases in all transcripts.

Table 3
Annotation of transcripts to six functional databases.

Database	Annotated Number	Transcript Ratio (%)	Genes Number	Gene Ratio (%)
NR	109,882	43.7	52,343	35.82
SwissProt	83,027	33.02	42,396	29.01
KEGG	104,244	41.46	49,877	34.13
KOG	70,428	28.01	35,931	24.59
PFAM	41,729	16.6	22,252	15.23
GO	79,419	31.59	38,365	26.25
Total	114,755	45.64	56,886	38.93

annotation, these genes were assigned to 58 GO categories at the second level (Fig. S2) and 46 KEGG categories (Fig. S3). Cellular process (22,298 genes, 58.12%), Metabolic process (22,284 genes, 58.08%), and single-organism process (15,280 genes, 39.83%) accounted for the top3 term in biological process category, while binding (21,100 genes, 54.00%) and catalytic activity (19,876 genes, 51.81%) were dominant in the molecular function category. In relation to pathway classification, genes were mainly distributed in Signal transduction (6057 genes, 12.14%), Carbohydrate metabolism (3535 genes, 7.09%), Translation (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 (qvalue < 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, CPPS1, 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 FACoAR1-encoding gene], “Starch and sucrose metabolism” [12 genes, including GH-catalytic, CAT-like, GT1, probable CCoAOMT, ThPPJ, GT20 and CWI3-encoding genes], “Stilbenoid, diarylheptanoid and gingerol biosynthesis” [4 genes, including CATL and probable CCoAOMT-encoding genes]. The top 10 KEGG pathways, including 4 significant 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], “Zeatin biosynthesis” [6 genes encoding UDPGT genes and CKX], “Glutathione metabolism” [10 genes encoding GST, RDRLL protein and GPX] and “Sesquiterpenoid and triterpenoid biosynthesis” [4 genes encoding PLOA/SOA; Table S3].

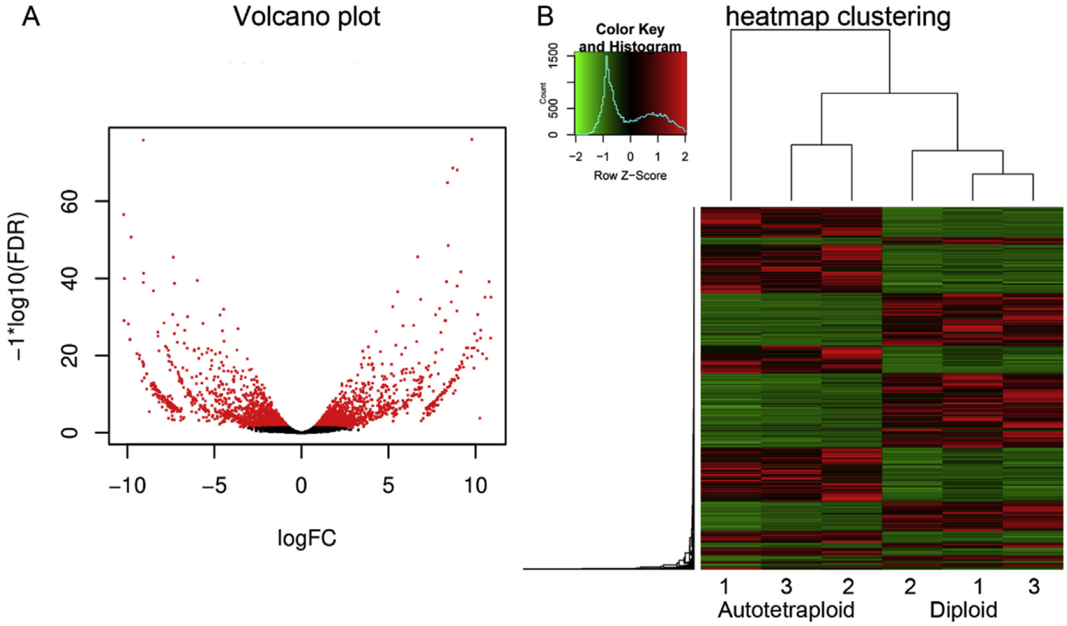

Fig. 3. DEGs and clustering analysis. A, volcano plot of DEGs. DEGs were predicted using PossionDis with the thresholds of fold change ≥1.5 and FDR ≤0.05. B, the heatmap clustering of DEGs in samples from autotetraploid and diploid *S. rebaudiana* leaves.

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 (67%) 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 differential expressed genes (P-value < 0.5), probably to be key enzymes in SGs biosynthesis, which were in agree with previous reports (Lucho et al., 2018; Bondarev et al., 2001). Further qRT-PCR analysis showed similar expression patterns as found in NGS data, where CPPS1 and UGT74G1 were significantly up-regulated in autotetraploids leaves (Fig. 5C). These results suggested that SGs biosynthesis pathway seemed to be activated in the autotetraploid *S. rebaudiana* leaves, which leading to the accumulation of SG contents in autotetraploid *S. rebaudiana* leaves obviously higher than diploid plants.

In order to uncover a comprehensive molecular mechanism about SGs biosynthesis, WGCNA was performed and modules associated with SGs biosynthesis were got subsequently. Overall, 32,533 genes with FPKM ≥0.5 were employed and clustered into 34 WGCNA modules, 7 modules (blue, brown, green, magenta, red, turquoise, and yellow, overall 25,441 genes) included at least 1 of the 15 SG biosynthesis-related genes were selected for follow-up functional enrichment analysis (Table S4). These module genes showed an almost overall up-regulated trend in autotetraploids regardless all genes or DEGs (Fig. 6). Furthermore, these module DEGs were associated with GO biological processes including protein ubiquitination and catabolic activity, photosynthesis, DNA replication and nucleosome assembly, flavonoid and secondary metabolic process, plant morphogenesis, plant growth, and signal transduction in plant, respectively (Fig. 7). These results suggested that polyploidy promote the biomass accumulation indirectly via various biological processes, in addition to directly regulation in MEP pathway.

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 interviewed 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 isoform X3], “D-Glutamine 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 “Tropane, 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; Lucía 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

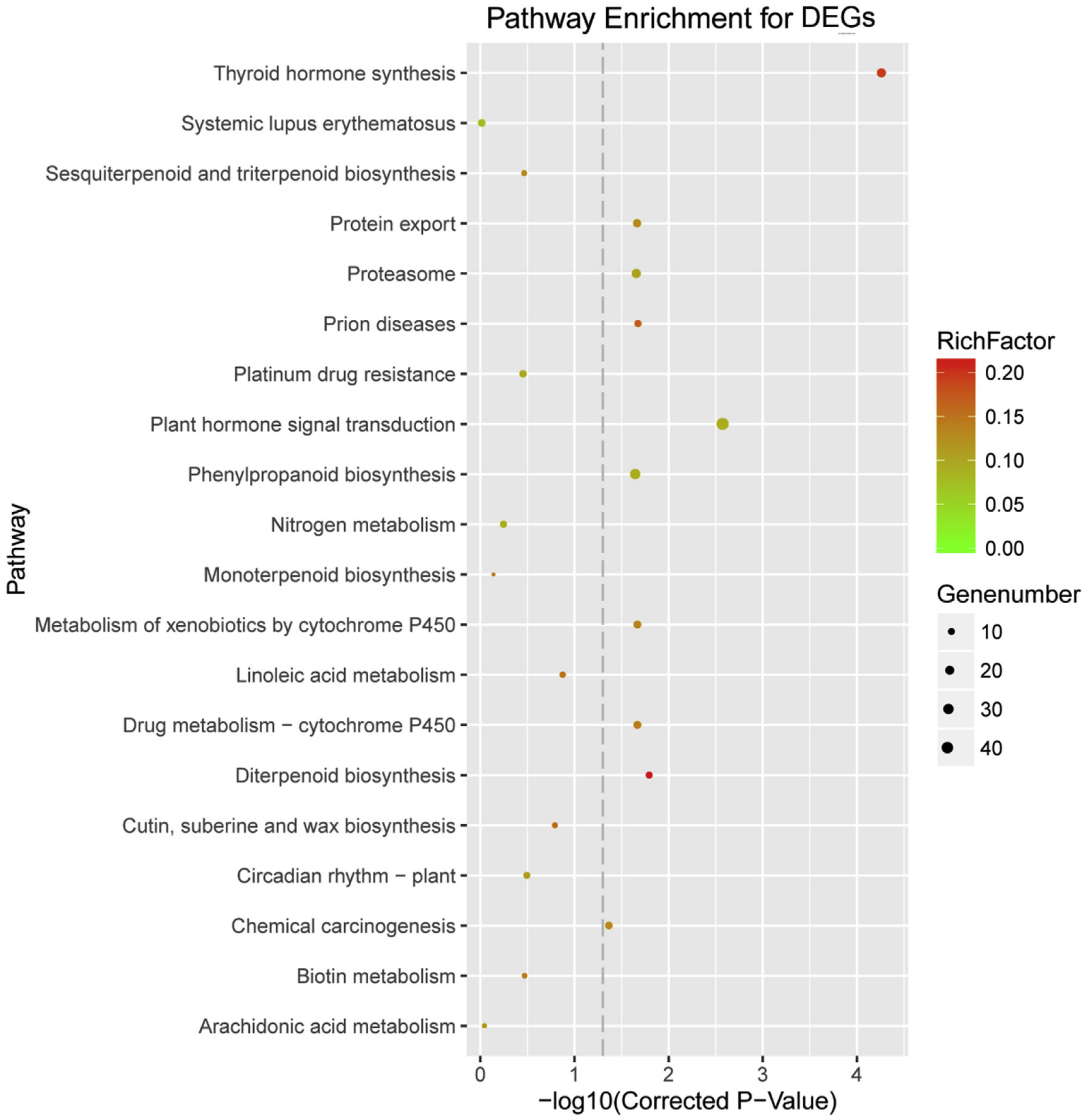

Fig. 4. Pathways associated significantly altered genes in autotetraploid *S. rebaudiana* comparing with diploid. The top 20 pathways. Notes indicate gene numbers. The larger the note, the higher gene number.

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

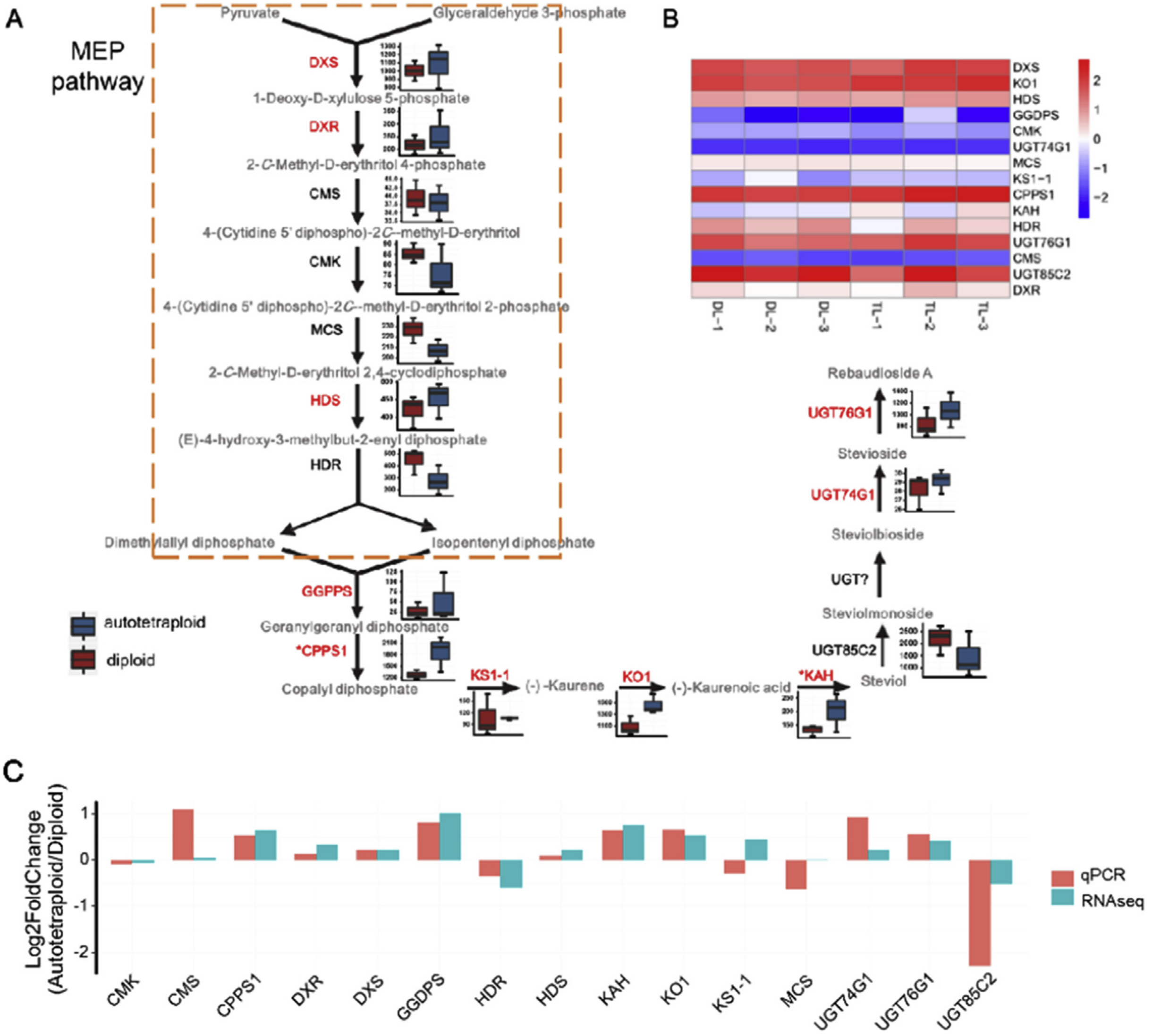

Fig. 5. Changed profiles of fifteen genes involved in steviol glycosides (SGs) biosynthesis. A, Diagrammatic representation and expression distribution of gene (s) involved in SGs biosynthesis. Genes in red represented up-regulated in autotetraploids leaves with a range of 15%–100%. Asterisk represented differential expressed with P-value < 0.05. B, heatmap analysis of 15 genes. C, relative expression levels of 15 genes in autotetraploid and diploid S. rebaudiana plants by RNA-seq and qRT-PCR, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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 decreased 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; Lucía 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

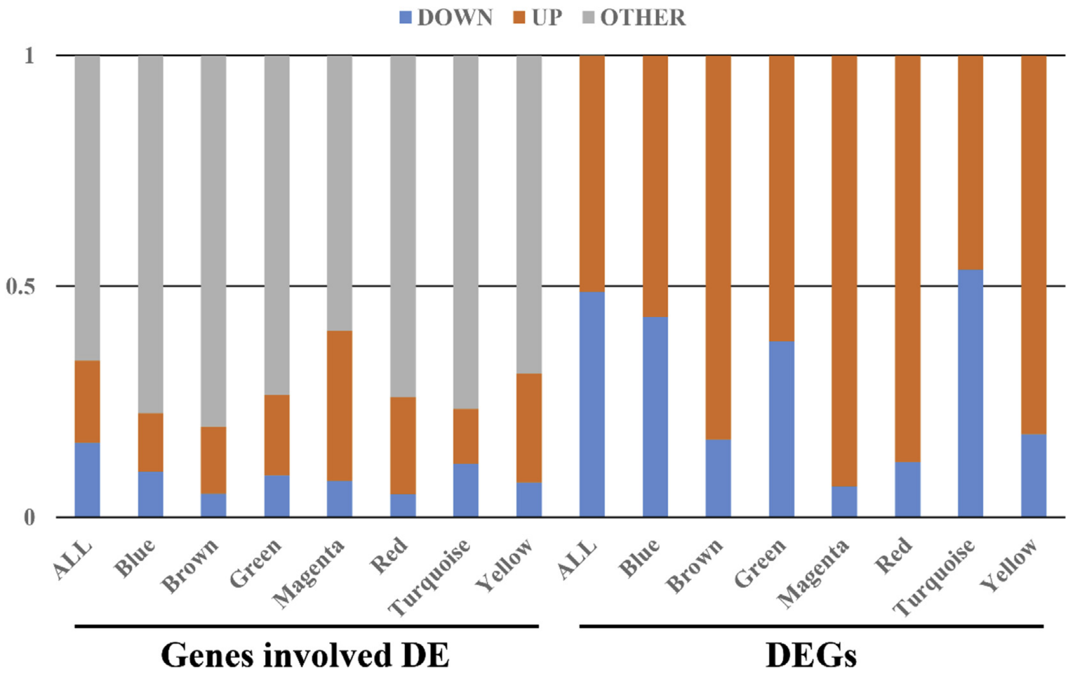

Fig. 6. Expression patterns of WGCNA modules genes associated with steviol glycosides biosynthesis distributed among all genes and all DEGs. Almost all the WGCNA modules genes showed an overall up-regulated trend in autotetraploids compared to diploids.

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

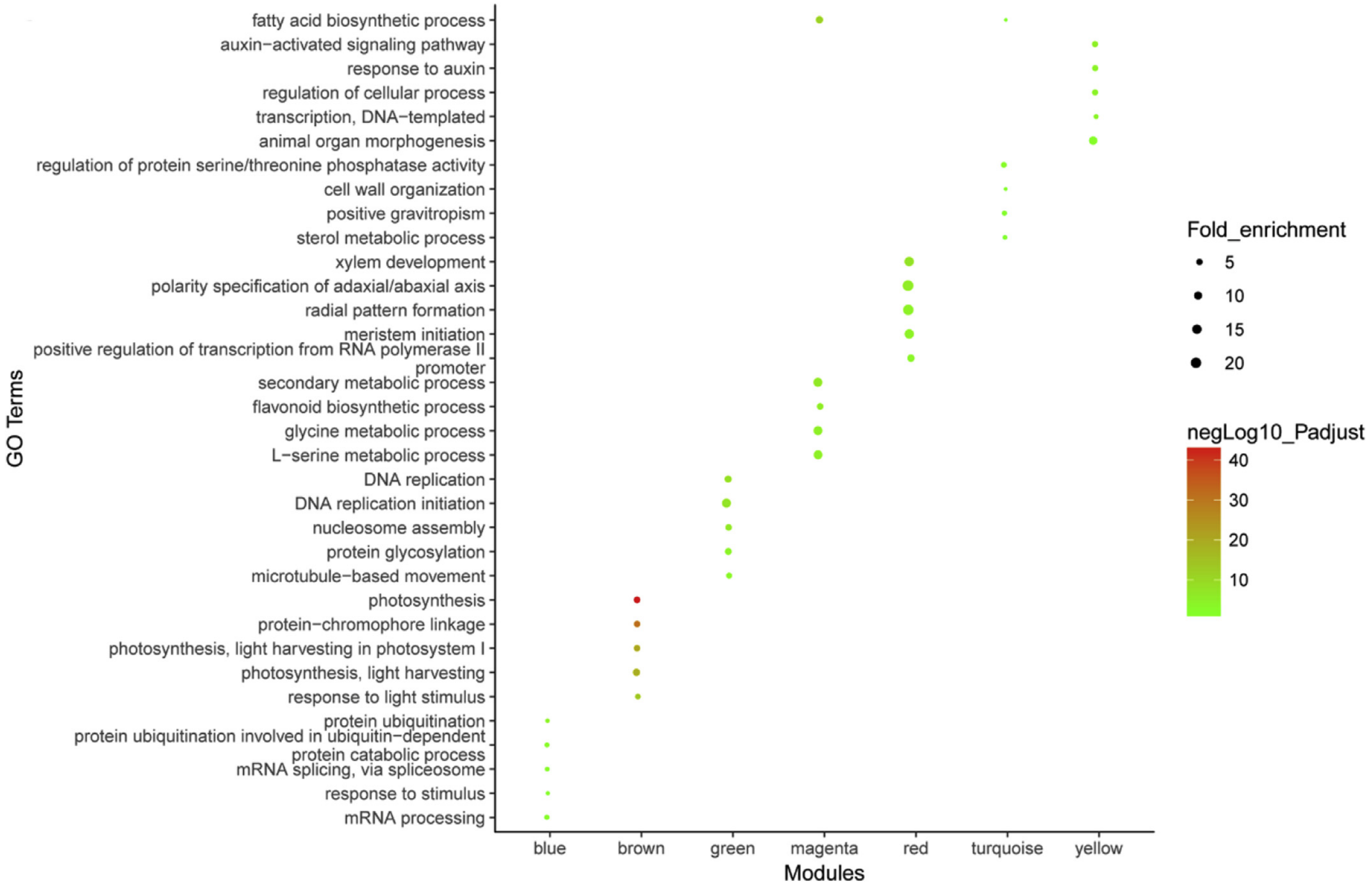

Fig. 7. GO biological processes associated with steviol glycosides biosynthesis related genes in WGCNA modules. The larger the node, the more gene number.

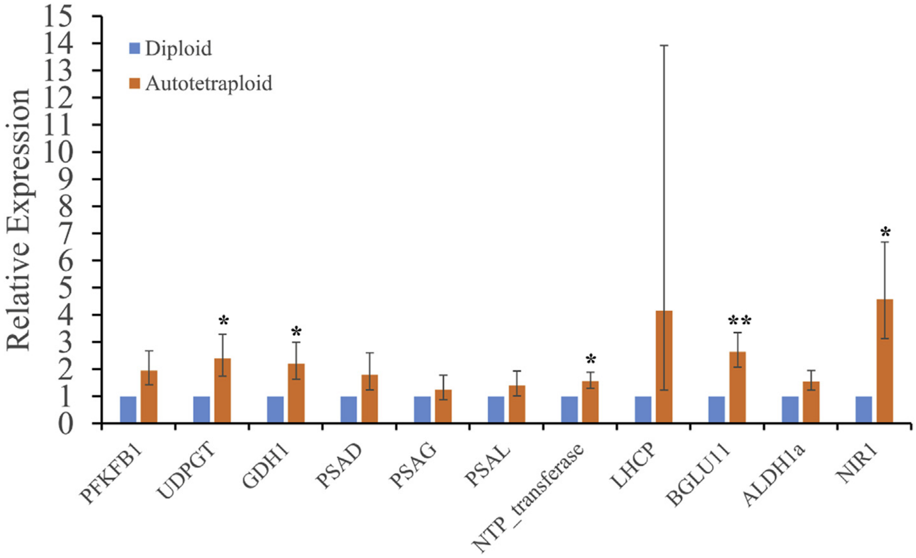

Fig. 8. Histograms representing the expression pattern of 11 selected resistance genes identified by qRT-PCR. **t*-test P value < 0.05; ** *t*-test P value < 0.01.

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% $HgCl_2$, 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 (1500–2000 Lx). Then, seedlings were transferred to 1/2 MS medium (containing 0.1 mg/L NAA and 0.1 mg/L IBA) for rooting. Finally, seedlings with roots were transplanted into nutritional soil.

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 Na_2EDTA, 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, stevioside, 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 Shidmadzu 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, snapped 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) (Haas 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 $2^{-\triangle\triangle Ct}$ 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 ploidy-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
DEG	differentially expression gene
T2D	type 2 diabetes mellitus
RA	rebaudioside A
RB	rebaudioside B
MEP	methyl erythritol 4-phosphate
DXS	1-deoxy-D-xylulose 5-phosphate synthase
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
CMS	4-diphosphocytidyl-2-C-methyl-D-erythritol synthase
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/DMAPP synthase
GGDPS	Geranyl geranyl diphosphate synthase
CPPS1	copalyl pyrophosphate synthase
KS1-1	Kaurene synthase
KO1	ent-kaurene oxidase CYP701A5
KAH	ent-kaurenoic acid 13-hydroxylase
UGT85C2	UDP glucosyltranserase – 85C2
UGT74G1	UDP glucosyltranserase – 74G1
UGT76G1	UDP glucosyltranserase – 76G1
UDPGT	UDP-glucuronosyl/UDP-glucosyltransferase
KSL	kaurene synthase like protein
GA3ox	gibberellin 3-oxidase
GH	glycoside hydrolase
GH-catalytic	glycoside hydrolase, catalytic domain-containing protein
CAT	chloramphenicol acetyltransferase
CATL	chloramphenicol 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 hydroxylase
CYP450	cytochrome P450
FACoAR1	gland-specific fatty acyl-CoA reductase 1
GT	glycosyl transferase, family
ThPPJ	trehalose phosphate phosphatase J
CWI3	cell wall invertase
F3′H	flavonoid 3′-hydroxylase
PLAT2	lipase/lipooxygenase, PLAT/LH2 family protein
CKX	cytokinin oxidase
GST	glutathione S-transferase
RDRLL	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 aminotransferase
HAS	Heme A synthase
Cox15	cytochrome C Oxidase Assembly Homolog
JMT	jasmonic acid carboxyl methyltransferase
GELP	GDSL esterase/lipase

References

Abbas Momtazi-Borojeni, A., Esmaeili, S.-A., Abdollahi, E., Sahebkar, A., 2017. A review on the pharmacology and toxicology of steviol glycosides extracted from *Stevia rebaudiana*. Curr. Pharmaceut. Des. 23, 1616–1622.

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. 90, 7327–7331.

Barriocanal, L.A., Palacios, M., Benitez, G., Benitez, S., Jimenez, J.T., Jimenez, N., Rojas, V., 2008. Apparent lack of pharmacological effect of steviol glycosides used as sweeteners in humans. A pilot study of repeated exposures in some normotensive and hypotensive individuals and in Type 1 and Type 2 diabetics. Regul. Toxicol. Pharmacol. 51, 37–41.

Behroozi, P., Baghizadeh, A., Saei, A., Kharazmi, S., 2017. Quantitative analysis of uridine diphosphate glycosyltransferase UGT85C2, UGT74G1 and UGT76G1 genes expression in *Stevia rebaudiana* under different irrigations. Russ. J. Plant Physiol. 64, 67–72.

Bondarev, N., Reshetnyak, O., Nosov, A., 2001. Peculiarities of diterpenoid steviol glycoside production in in vitro cultures of *Stevia rebaudiana* Bertoni. Plant Sci. 161, 155–163.

Bondarev, N.I., Sukhanova, M.A., Reshetnyak, O.V., Nosov, A.M., 2003. Steviol glycoside content in different organs of *Stevia rebaudiana* and its dynamics during ontogeny. Biol. Plant. 47 (2), 261–264.

Brandle, J., Telmer, P., 2007. Steviol glycoside biosynthesis. Phytochemistry 68, 1855–1863.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST+: architecture and applications. BMC Bioinf. 10, 421.

Carakostas, M., Curry, L., Boileau, A., Brusick, D., 2008. Overview: the history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. Food Chem. Toxicol. 46, S1–S10.

Chatsudthipong, V., Muanprasat, C., 2009. Stevioside and related compounds: therapeutic benefits beyond sweetness. Pharmacol. Ther. 121, 41–54.

Chepyshko, H., Lai, C.-P., Huang, L.-M., Liu, J.-H., Shaw, J.-F., 2012. Multifunctionality and diversity of GDSL esterase/lipase gene family in rice (*Oryza sativa* L. japonica) genome: new insights from bioinformatics analysis. BMC Genomics 13, 309.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M.Blast2GO., 2005. A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676.

Dong, X., Yi, H., Han, C.-T., Nou, I.-S., Hur, Y., 2016. GDSL esterase/lipase genes in *Brassica rapa* L.: genome-wide identification and expression analysis. Mol. Genet. Genom. 291, 531–542.

Fu, L., Niu, B., Zhu, Z., Wu, S., Li, W., 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28, 3150–3152.

Gamir, J., Darwiche, R., van't Hof, P., Choudhary, V., Stumpe, M., Schneiter, R., Mauch, F., 2017. The sterol-binding activity of PATHOGENESIS-RELATED PROTEIN 1 reveals the mode of action of an antimicrobial protein. Plant J. 89, 502–509.

Gao, R., Wang, H., Dong, B., Yang, X., Chen, S., Jiang, J., Zhang, Z., Liu, C., Zhao, N., Chen, F., 2016. Morphological, genome and gene expression changes in newly induced autopolyploid *Chrysanthemum lavandulifolium* (fisch. Ex trautv.) makino. Int. J. Mol. Sci. 17 (10), 1690.

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat. Protoc. 8, 1494.

He, Y., Sun, Y., Zheng, R., Ai, Y., Cao, Z., Bao, M., 2016. Induction of tetraploid male sterile tagetes erecta by colchicine treatment and its application for interspecific hybridization. Hortic. Plants J. 2, 284–292.

Hollister, J.D., Arnold, B.J., Svedin, E., Xue, K.S., Dilkes, B.P., Bomblies, K., 2012. Genetic adaptation associated with genome-doubling in autotetraploid *Arabidopsis arenosa*. PLoS Genet. 8, e1003093.

Humphrey, T.V., Richman, A.S., Menassa, R., Brandle, J.E., 2006. Spatial organisation of four enzymes from *Stevia rebaudiana* that are involved in steviol glycoside synthesis. Plant Mol. Biol. 61 (1–2), 47–62.

Jaskani, M.J., Kwon, S.W., Kim, D.H., 2005. Comparative study on vegetative, reproductive and qualitative traits of seven diploid and tetraploid watermelon lines. Euphytica 145, 259–268.

Langfelder, P., Horvath, S., 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinf. 9, 559.

Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinf. 12, 323.

Liu, S., Chen, S., Chen, Y., Guan, Z., Yin, D., Chen, F., 2011. In vitro induced tetraploid of *Dendranthema nankingense* (Nakai) Tzvel. shows an improved level of abiotic stress tolerance. Sci. Hortic. 127, 411–419.

Lu, B., Pan, X., Zhang, L., Huang, B., Sun, L., Li, B., Li, B., Zheng, S., Yu, X., Ding, R., Chen, W., 2006. A genome-wide comparison of genes responsive to autopolyploidy inIsatis indigotica using *Arabidopsis thaliana* Affymetrix genechips. Plant Mol. Biol. Report. 24 (2), 197–204.

Lucho, S.R., do Amaral, M.N., Milech, C., Ferrer, M.Á., Calderón, A.A., Bianchi, V.J., Braga, E.J.B., 2018. Elicitor-induced transcriptional changes of genes of the steviol glycoside biosynthesis pathway in *Stevia rebaudiana* bertoni. J. Plant Growth Regul. 1–15.

Lucía, G.R., Jesica, I., Andrea, C., Verónica, B., Paula, B., Sandra, P.-Á., Alejandro, E., 2015. A protocol for the in vitro propagation and polyploidization of an interspecific hybrid of *Glandularia* (*G. peruviana* × *G. scrobiculata*). Sci. Hortic. 184, 46–54.

Madani, H., Hosseini, B., Dehghan, E., Rezaei-chiyaneh, E., 2015. Enhanced production of scopolamine in induced autotetraploid plants of *Hyoscyamus reticulatus* L. Acta Physiol. Plant. 37, 55.

Martin, S.L., Husband, B.C., 2013. Adaptation of diploid and tetraploid Chamerion angustifolium to elevation but not local environment. Evolution 67, 1780–1791.

Mohamed, A.A., Ceunen, S., Geuns, J.M., Van den Ende, W., De Ley, M., 2011. UDP-dependent glycosyltransferases involved in the biosynthesis of steviol glycosides. J. Plant Physiol. 168, 1136–1141.

Mu, H.-Z., Liu, Z.-J., Lin, L., Li, H.-Y., Jiang, J., Liu, G.-F., 2012. Transcriptomic analysis of phenotypic changes in birch (*Betula platyphylla*) autotetraploids. Int. J. Mol. Sci. 13, 13012–13029.

Naranjo, M.A., Forment, J., RoldÁN, M., Serrano, R., Vicente, O., 2006. Overexpression of *Arabidopsis thaliana* LTL1, a salt-induced gene encoding a GDSL-motif lipase, increases salt tolerance in yeast and transgenic plants. Plant Cell Environ. 29, 1890–1900.

Onakpoya, I.J., Heneghan, C.J., 2015. Effect of the natural sweetener, steviol glycoside, on cardiovascular risk factors: a systematic review and meta-analysis of randomised clinical trials. Eur. J. Prev. Cardiol. 22, 1575–1587.

Philippaert, K., Pironet, A., Mesuere, M., Sones, W., Vermeiren, L., Kerselaers, S., Pinto, S., Segal, A., Antoine, N., Gysemans, C., 2017. Steviol glycosides enhance pancreatic beta-cell function and taste sensation by potentiation of TRPM5 channel activity. Nat. Commun. 8, 14733.

Pignatta, D., Dilkes, B.P., Yoo, S.Y., Henry, I.M., Madlung, A., Doerge, R.W., Chen, Z.J., Comai, L., 2010. Differential sensitivity of the *Arabidopsis thaliana* transcriptome and enhancers to the effects of genome doubling. New Phytol. 186 (1), 194–206.

Preuß, A., Augustin, C., Figueroa, C.R., Hoffmann, T., Valpuesta, V., Sevilla, J.F., Schwab, W., 2014. Expression of a functional jasmonic acid carboxyl methyltransferase is negatively correlated with strawberry fruit development. J. Plant Physiol. 171, 1315–1324.

Purkayastha, S.; Markosyan, A.; Martin, J.; Petit, M. Stevia Composition to Improve Sweetness and Flavor Profile. Google Patents: 2016.

Renny-Byfield, S., Wendel, J.F., 2014. Doubling down on genomes: polyploidy and crop plants. Am. J. Bot. 101, 1711–1725.

Richman, A.S., Gijzen, M., Starratt, A.N., Yang, Z., Brandle, J.E., 1999. Diterpene synthesis in *Stevia rebaudiana*: recruitment and up-regulation of key enzymes from the gibberellin biosynthetic pathway. Plant J. 19 (4), 411–421.

Robinson, M.D., McCarthy, D.J., Smyth, G.K. edgeR., 2010. A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140.

Sakhanokho, H.F., Islam-Faridi, M.N., 2014. Spontaneous autotetraploidy and its impact on morphological traits and pollen viability in *Solanum aethiopicum*. Hortscience 49, 997–1002.

Schumann, M.J., Zeng, Z.-B., Clough, M.E., Yencho, G.C., 2017. Linkage map construction and QTL analysis for internal heat necrosis in autotetraploid potato. Theor. Appl. Genet. 130, 2045–2056.

Seo, H.S., Song, J.T., Cheong, J.-J., Lee, Y.-H., Lee, Y.-W., Hwang, I., Lee, J.S., Do Choi, Y., 2001. Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. 98, 4788–4793.

Singh, G., Singh, G., Singh, P., Parmar, R., Paul, N., Vashist, R., Swarnkar, M.K., Kumar, A., Singh, S., Singh, A.K., 2017. Molecular dissection of transcriptional reprogramming of steviol glycosides synthesis in leaf tissue during developmental phase transitions in *Stevia rebaudiana* Bert. Sci. Rep. 7, 11835.

Song, M.S., Kim, D.G., Lee, S.H., 2005. Isolation and characterization of a jasmonic acid carboxyl methyltransferase gene from hot pepper (*Capsicum annuum* L.). J. Plant Biol. 48, 292–297.

Stupar, R.M., Bhaskar, P.B., Yandell, B.S., Rensink, W.A., Hart, A.L., Ouyang, S., Veilleux, R.E., Busse, J.S., Erhardt, R.J., Buell, C.R., 2007. Phenotypic and transcriptomic changes associated with potato autopolyploidization. Genetics 176, 2055–2067.

van Loon, L.C., Rep, M., Pieterse, C.M., 2006. Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44, 135–162.

Wang, J., Tian, L., Madlung, A., Lee, H.-S., Chen, M., Lee, J.J., Watson, B., Kagochi, T., Comai, L., Chen, Z.J., 2004. Stochastic and epigenetic changes of gene expression in Arabidopsis polyploids. Genetics 167, 1961–1973.

Wu, Y., Li, W., Dong, J., Yang, N., Zhao, X., Yang, W., 2013. Tetraploid induction and cytogenetic characterization for Clematis heracleifolia. Caryologia: Int. J. Cytol. Cytosystematics Cytogenet. 66, 215–220.

Wu, J., Shahid, M.Q., Guo, H., Yin, W., Chen, Z., Wang, L., Liu, X., Lu, Y., 2014. Comparative cytological and transcriptomic analysis of pollen development in autotetraploid and diploid rice. Plant Reprod. 27, 181–196.

Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., Kong, L., Gao, G., Li, C.Y., Wei, L., 2011. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res. 39, W316–W322. https://doi.org/10.1093/nar/gkr483.

Yadav, A.K., Singh, S., Dhyani, D., Ahuja, P.S., 2011. A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)]. Can. J. Plant Sci. 91, 1–27.

Yan, K., Wu, C., Zhang, L., Chen, X., 2015. Contrasting photosynthesis and photoinhibition in tetraploid and its autodiploid honeysuckle (*Lonicera japonica* Thunb.) under salt stress. Front. Plant Sci. 6, 227.

Yang, P.-M., Huang, Q.-C., Qin, G.-Y., Zhao, S.-P., Zhou, J.-G., 2014. Different drought-stress responses in photosynthesis and reactive oxygen metabolism between autotetraploid and diploid rice. Photosynthetica 52, 193–202.

Yang, Y., Huang, S., Han, Y., Yuan, H., Gu, C., Wang, Z., 2015. Environmental cues induce changes of steviol glycosides contents and transcription of corresponding biosynthetic genes in *Stevia rebaudiana*. Plant Physiol. Biochem. 86, 174–180.

Yu, Z., Haberer, G., Matthes, M., Rattei, T., Mayer, K.F., Gierl, A., Torres-Ruiz, R.A., 2010. Impact of natural genetic variation on the transcriptome of autotetraploid *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. 107, 17809–17814.

Yu, G., Wang, L.-G., Han, Y., He, Q.-Y., 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS A J. Integr. Biol. 16, 284–287.

Yu, L.-X., Liu, X., Boge, W., Liu, X.-P., 2016. Genome-wide association study identifies loci for salt tolerance during germination in autotetraploid alfalfa (*Medicago sativa* L.) using genotyping-by-sequencing. Front. Plant Sci. 7, 956.

Zhang, X., Deng, M., Fan, G., 2014. Differential transcriptome analysis between *Paulownia fortunei* and its synthesized autopolyploid. Int. J. Mol. Sci. 15, 5079–5093.

Zhou, Y., Kang, L., Liao, S., Pan, Q., Ge, X., Li, Z., 2015. Transcriptomic analysis reveals differential gene expressions for cell growth and functional secondary metabolites in induced autotetraploid of Chinese woad (*Isatis indigotica* Fort.). PLoS One 10, e0116392.

Zhu, H., Liu, W., 2018. Progress on salt resistance in autopolyploid plants. Hereditas 40, 315–326.