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Karyotype analysis and genetic variation of a mutant in *Siraitia grosvenorii*

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Abstract This study analyzed the differences in karyotype and genetic variation between a mutant and wild-type *Siraitia grosvenorii*. Genetic variation included changes in genome and gene expression by SRAP molecular markers. Results showed that wild-type *S. grosvenorii* was diploid, with a chromosome number of $2n = 2x = 28$, whereas the mutant was tetraploid with a chromosome number of $2n = 4x = 56$. 4573 DNA bands were obtained using 189 different primer combinations, 577 of which were polymorphic, averaging 3.1 bands for each primer pair, while 1998 pairs were identical. There were no apparent differences on bands amplified by most primer pairs. After comparing the diploid and tetraploid strains, the data generally indicated that the polymorphism would be quite low. 2917 cDNA bands were generated using 133 primer combinations, and stable and clearly differential fragments were sorted out, cloned and sequenced. Ninety-two differentially expressed fragments were successfully sequenced. Sequence analysis showed that most fragments had significant homologous nucleotide sequences with resistant to stress and photosynthesis genes, including ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxykinase, pyruvate kinase, peroxisomal membrane transporter, NBS-LRR type resistance protein, protein phosphatase and others. The results revealed that the tetraploid strain has more resistant and photosynthesis ability than its diploid relatives, which providing reference information and resources for molecular breeding and seedless Luohanguo.

Keywords Gene expression · Tetraploid · Triploid · Seedless · SRAP

Introduction

Siraitia grosvenorii (Swingle) C. Jeffrey, belonging to the genus *Siraitia Merr* under the family Cucurbitaceae, is a precious and economically important species endemic to southern China that and has been cultivated for several centuries. The fruits of *S. grosvenorii*, called Luohanguo, are used for food, beverages and traditional Chinese medicine. The fruit has evident effects on dry cough, sore throat, extreme thirst and constipation [1]. The major components of Luohanguo are mogrosides, a group of terpene glycosides, estimated to be about 300 times as sweet as sucrose [2]. One of the mogroside, mogroside V, is 400 times sweeter than sucrose [3]. Moreover, Luohanguo extracts have chemopreventive and antioxidant properties. Its non-caloric properties and effects on the cancer chemopreventive and antioxidant, being used as sweetening are being used as sweetening agents for the patients with diabetes mellitus, adiposis, hypertension, heart disease and so on. Luohanguo products have been approved as dietary supplements in Japan, the United States, New Zealand and Australia [4]. With the rapid rise in market demand, Luohanguo extracts have increased rapidly from two tons in 2002 to 60 tons in 2007, becoming one of the fastest growing traditional Chinese medicine extracts.

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Our group isolated a novel male mutant plant (M038) from hybrids (M036) of 'Bolin' and 'PinzhongND'. The mutant exhibited growth vigor, larger body and increased organ size compared to wild type. We then used pollen of M038 to pollinate 'Nongyuan'(a main cultivar), and the following year fruits of female hybrids (F050) were seedless (Fig. 1). Mogrosides only exist in flesh, occupying 15% of the dry weigh, whereas seeds constitute 70% of the weight but contain no mogrosides. Thus, seedless Luohanguo will no doubt increase the utilization and extraction rate of mogrosides. Therefore, mutant M038 and hybrid seedless Luohanguo are of great research value. M038 and F050 possesses distinct differences compared with wild type based on biological characters identified through our investigation: (1) M038 phenotypes include their strong growth potential. They are larger, have a thicker stem, wider and longer leaves, increased leaf area, bigger flowers and enhanced biomass. Both M038 and hybrid F050 have strong growth advantages as their flowering time, growing time and maturing time are all delayed. (2) The flower organ of M038 is particularly wider, while pollen is seriously aborted. Additionally, pollen quantity is less, while the germination rate of pollen is low. Most pollen is irregular and abnormal, shriveled and hollow. (3) Soluble sugar, glucose, mogroside, vitamin C and protein contents of F050 seedless fruits are all higher than in other main cultivars.

In this study, we carried out preliminary studies on karyotype analysis and the molecular mechanism of mutant M038. The results will provide reference information and resources for molecular breeding of seedless Luohanguo.

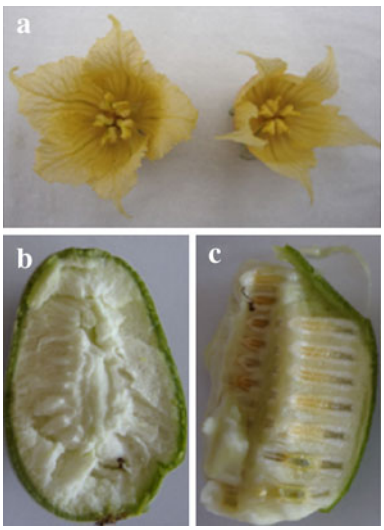

Fig. 1 **a** Flowers of *S. grosvenorii* mutant M038 and wild-type M036; **b** Fruits of seedless Luohanguo F050; **c** Fruits of normal Luohanguo

Materials and methods

Mutant M038, wild-type M036 and hybrid F050 were obtained from the Guangxi Branch Institute, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College.

Karyotype analysis

Root tips 0.5–1 cm in length were collected from young plants and pretreated with saturated aqueous solution of p-dichlorobenzene at room temperature for 3 h before they were fixed in Carnoy (glacial acetic acid: absolute ethanol 1:3) for 1 h. The tips were washed in distilled water, hydrolyzed in 1 mmol/l HCL at 45°C for 45 min, stained in phenol-fuchsin solution and squashed. The designation used to describe the karyotypes follows Li and Chen [5]. Karyotype symmetry was classified according to Stebbins [6].

DNA extraction

Genomic DNA was extracted from fresh leaves using the cetyltrimethylammonium bromide procedure [7]. Extracted DNA samples were dissolved in TE buffer and visualized after electrophoresis on 0.8% 1× TAE agarose gels. DNA purity and concentration was measured with a UV spectrophotometer and Nanodrop 8000. The DNA was adjusted to a final concentration of 30 ng/μl and stored at −20°C until use.

RNA extraction and cDNA synthesis

Total RNA was isolated from pooled fresh leaves using the TRIzol reagent (Invitrogen, USA). All the RNAs were with treated by RNA-free DNase I and purified. RNA purity and concentration was measured with a UV spectrophotometer and Nanodrop 8000. M-MLV (RNase H) reverse transcriptase was used to synthesize cDNA following the manufacturer's protocol. Two technical replicates were performed.

SRAP analysis

A total of 196 different primer combinations were employed using 14 forward primers and 14 reverse primers (Supplementary Table 1). The primers sequences were synthesized according to Li and Quiros [8]. The PCR amplification for SRAP analysis were performed as follow: 94°C for 5 min, followed by 5 cycles at 94°C for 45 s, 35°C for 45 s, and 72°C for 45 s; 35 cycles at 94°C for 45 s, 50°C for 45 s, 72°C for 45 s and a final extension at 72°C for 7 min. The PCR products were analyzed using the

QIAxcel capillary electrophoresis system, an automated analyzer offering high resolution and short analysis time. The system includes a device, cartridge, BioCalculator Analysis software and computer, and it applies capillary electrophoresis and fluorescence technology (Fig. 2).

Isolation and sequencing of differential fragments

The amplification products of the bands of interest were run on a 6% polyacrylamide gel at 45 W for 1.5 h in 1× TBE buffer and silver stained, and the bands were cut from the gel and eluted in 50 μl of sterile double distilled water. About 5 μl of the aliquot was reamplified using the same primer pairs and the same PCR conditions. The PCR products were ran in a 2% agarose gel and the bands were isolated and eluted using the gel extraction kit. Eluted fragments were cloned into plasmid pMD19-T propagated in *E. coli* DH5 cells and sent to Sangon biotechnology for sequencing.

Sequences analysis

The resulting sequences were compared to nucleotide and protein sequences in publicly available databases using BLAST sequence alignments. The functions of known genes identified by BLASTN and BLASTX searches were classified according to their putative functions.

Results and discussion

Karyotype analysis

The karyotypes of three materials studied are presented (Fig. 3). The chromosome numbers of M036, M038 and F050 were $2n = 2x = 28$, $2n = 4x = 56$ and $2n = 3x = 42$, respectively. The results showed that wild-type M036 was a diploid strain, and the chromosome numbers $2n = 2x = 28$ were consistent with what has been previously been published on *S. grosvenorii* [9–11]. Mutant M038 was a tetraploid, and hybrid F050 was a triploid. Furthermore, M038 was an autopolyploid occurring within a species, relative to allopolyploidy following interspecific hybridization [6, 12]. To our knowledge, this is the first report on karyotype and molecular studied of polyploids in *S. grosvenorii*.

Genomic changes

Polyploidy, including autopolyploidy and allopolyploidy, may trigger changes in genome structure and gene expression [13]. At present, studies on polyploid plants have mainly concentrated on polyploid *Arabidopsis*, *Brassica*, *Triticum*, *Gossypium*, *Nicotiana*, *Senecio*, *Spartina*, *Tragopogon* and *Triticale* [14–28]. Most studies on polyploidization have focused on allopolyploid species

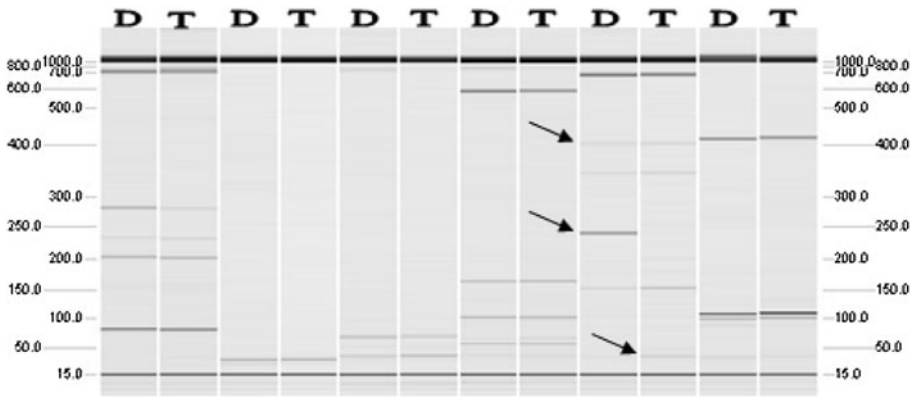

Fig. 2 QIAxcel capillary electrophoresis image of SRAP analysis. The *top arrow* indicates a band present in both the diploid and tertaploid strains; The *middle arrow* indicates a band present in the diploid strain but absent in the tetraploid one; The *bottom arrow* indicates a band present in the tetraploid strain but absent in the diploid; The D represents diploid M036; T represents tetraploid M038

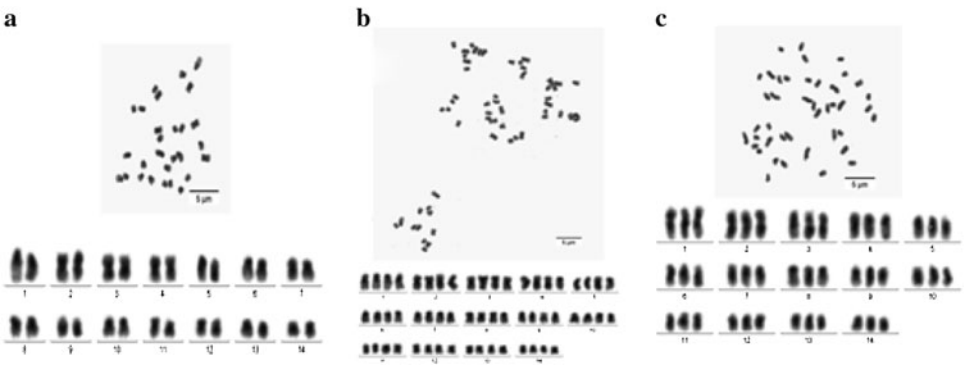

Fig. 3 Karyotypes of M036, M038 and F050. **a** M036 ($2n = 2x = 28$); **b** M038 ($2n = 4x = 56$); **c** F050 ($2n = 3x = 42$)

[17, 23, 28–31], whereas documents on autopolyploids are less [31–35]. Only a few studies dedicated to elucidating the consequences of autopolyploidization have been reported [34, 36–38].

A total of 196 SRAP primer combinations were used for DNA amplifications, nine combinations of which failed to amplify banding patterns. 189 SRAP primer combinations generated 4573 bands, 577(12.6%) of which were polymorphic while 1998 pairs (87.4%) were identical. Fragment bands obtained were in the size range of 100–800 bp, while the number of bands amplified by each primer combination ranged from 6 (ME11a-OD3) to 19 (Me11-SA4), with a mean of 12.1. Moreover, The number of polymorphic bands for each primer combination varied from 0 (ME4-SA4) to 5 (Me9-Em18a), with a mean of 3.1. Based on the percentage of polymorphic bands, the levels of polymorphism ranged from 0 (Me12-Em10) to 37.6% (PM8-Me10).

When chromosome doubling, genome structure and sequences have changed, it leads to differences and polymorphisms among diploid and tetraploid strains. According to conventional theory, It is expected that changes can not be observed during autopolyploidization. Our data also support general predictions. Only a small range of changes were observed in the genomes of tetraploid M038 compared to diploid M036. There were no apparent differences in the bands of M036 and M038 amplified by most primer pairs. The results generally indicated that the genetic diversity would be quite low between the diploid and tetraploid strains of *S. grosvenorii*.

Expression changes

Molecular results suggested that the rapid genome changes could accelerate evolutionary processes, and this may partly account for the appearance of many novel phenotypes. Further studies should focus on the characterization of the changed sequences and gene expression. Results of a wild autopolyploid sunflower series revealed that autopolyploidy does not appear to induce silencing or novel gene expression [39]. Addtionally, microarray analysis detected few changes associated with polyploidization [33]. The latest analysis of the transcriptome in an inbred maize ploidy series found frequent but low-level changes [40]. However, some articles demonstrated that autopolyploids also display differences in gene expression relative to diploids [17, 41].

A total of 196 SRAP primer combinations were used for cDNA amplifications, 63 of which failed to amplify any banding patterns. A total of 133 SRAP primer combinations generated 2917 bands, 289 (9.9%) of which were polymorphic while 1313 pairs (90.1%) were identical. Fragment bands obtained were in the size range of 100–800 bp. Stable and clearly differentiated fragments were sorted out, cloned and sequenced. Ninety-two differentially expressed fragments were successfully sequenced, 77.2% of them were highly homologous to known genes (Supplementary Table 2), 9.8% were hypothetical genes and 13.0% were possible new genes as they had no significant similarity to known genes. The sequences encoding putative proteins were classified into 15 small groups based on previously reported gene functions and information from Gene Ontology (Fig. 4). These sequences provided useful and important molecular information for following studies on the expression changes between diploid and tetraploid of *S. grosvenorii*.

Natural autopolyploids and autopolyploids developed for agricultural purposes are often more vigorous and larger in size than their diploid relatives, indicating a fitness advantage and phenotypic superiority associated with higher ploidy [42]. Most studies on gene expression in polyploids have been based on phenotypic differences and related physiological characters, however, studies at the molecular level have not been widely carried out. Sequence analysis revealed that most fragments had significant homologous nucleotide sequence to photosynthesis, respiration and stress response genes. These genes included ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxykinase, pyruvate kinase, peroxisomal membrane transporter, NBS-LRR type resistance protein, protein phosphatase and others. The results, to some extent, indicated that the tetraploid of *S. grosvenorii* has more resistance to stress and photosynthesis ability than its diploid relatives. It provided molecular evidences for the phenomenon of

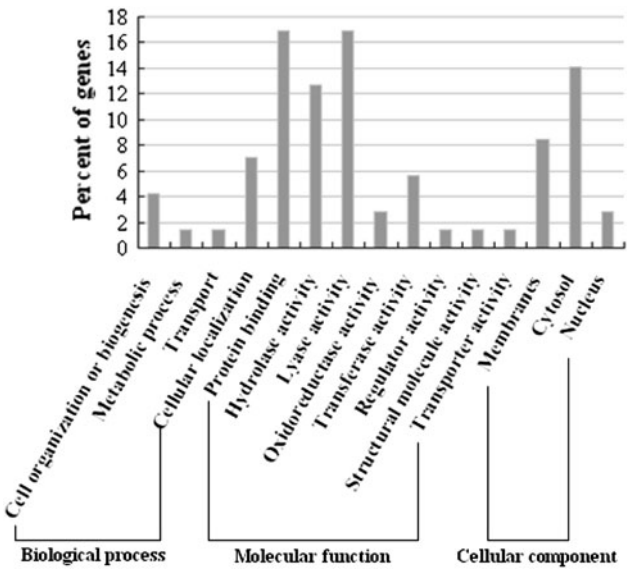

Fig. 4 Functional classification of differentially expressed fragments from cDNA-SRAP analysis based on gene functions and the GO annotation

polyploids exceeding their diploid relatives based on the phenotypic and biological features.

Furthermore, functional analysis showed that the gene-encoded proteins were involved in a broad range of biological pathways, including transporters, signal transduction, metabolism, transcription, protein synthesis, development, energy, etc. There proteins play important roles in regulating plant growth and development and include zinc finger protein, molecular chaperone, mitogen-activated kinase, transcription factor IWS1, transducin, endomembrane protein, porin, cellulose synthase, cytochrome P450, glycosyltransferase, oxidoreductase and other.

In summary, the interesting tetraploid mutant contains changes in genome structure and gene expression compared to the diploid strain. As this is the first study on tetraploid of *S. grosvenorii*., which will provide solid basis for future studies on tetraploid and hybrid triploid seedless Luohanguo. Future studies are planned to understand on more about genetic mechanism of triploid seedless Luohanguo.

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