Comparative study on cytogenetics and transcriptome between diploid and autotetraploid rice hybrids harboring double neutral genes

Lin Chen¹,²,³*, Haibin Guo²,⁴*, Shuling Chen¹,²,³, Huijing Yang¹,²,³, Fozia Ghouri¹,²,³, Muhammad Qasim Shahid¹,²,³*

¹ State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou, China, ² Guangdong Provincial Key Laboratory of Plant Molecular Breeding, South China Agricultural University, Guangzhou, China, ³ College of Agriculture, South China Agricultural University, Guangzhou, China, ⁴ Center of Experimental Teaching for Common Basic Courses, South China Agricultural University, Guangzhou, China

☯ These authors contributed equally to this work.
* qasim@scau.edu.cn

Abstract

Double pollen fertility neutral genes, Saⁿ and Sbⁿ, can control pollen sterility in interspecific (indica × japonica) rice hybrids, which has excellent potential to increase rice yield. Previous studies showed that polyploidy could increase the interaction of three pollen sterility loci, i.e., Sa, Sb and Sc, which cause pollen sterility in autotetraploid rice hybrids, and hybrid fertility could be improved by double neutral genes, Saⁿ and Sbⁿ, in autotetraploid rice hybrids. We compared cytological and transcriptome data between autotetraploid and diploid rice hybrid during meiosis and single microspore stages to understand the molecular mechanism of neutral genes for overcoming pollen sterility in autotetraploid rice hybrids, which harbored double neutral genes. Cytological results revealed that the double neutral genes resulted in higher pollen fertility (76.74%) and lower chromosomal abnormalities in autotetraploid hybrid than in parents during metaphase I, metaphase II, anaphase I and anaphase II. Moreover, autotetraploid rice hybrid displayed stronger heterosis than a diploid hybrid. Compared with diploid rice hybrid, a total of 904 and 68 differently expressed genes (DEGs) were identified explicitly in autotetraploid hybrid at meiosis and single microspore stages, respectively. Of these, 133 and 41 genes were detected in higher-parent dominance and transgressive up-regulation dominance, respectively, which were considered autotetraploid potential heterosis genes, including a meiosis-related gene (Os01g0917500, MSP1) and two meiosis specific-genes (Os07g0624900 and Os04g0208600). Gene Ontology (GO) and Kyoto Encyclopedia of Gene and Genomes pathway (KEGG) analysis revealed that DEGs significantly enriched in amino acid metabolism and photosynthesis metabolism. These results indicated that meiosis-specific and meiosis-related genes, and amino acids and photosynthesis metabolism-related genes contribute to higher pollen fertility in autotetraploid rice hybrid. This study provides a theoretical basis for
Funding: This work was supported by the NSFC to NCBI SRA database (SRA accession: PRJNA656120).

Abbreviations: qRT-PCR, Quantitative real-time polymerase chain reaction; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, Differentially expressed genes; DEGs

Introduction

Rice is one of the most key cereal crops which feeds more than half of the world’s population and is essential to food security. Hybrid rice has been recognized as one of the most suitable and sufficient technologies for increasing rice production. Asian cultivated rice mainly differentiated into indica and japonica after long domestication [1]. At present, indica-japonica hybrid rice has shown more substantial yield potential. Still, partial sterility has been a significant barrier to the utilization of the strong heterosis present in intersubspecific hybrids. One of the critical factors is the development of aborted pollen grains. So far, more than 50 loci controlling indica x japonica sterility have been identified [2, 3]. Pollen fertility of indica-japonica hybrids is controlled by at least six loci (Sa, Sb, Sc, Sd, Se and Sf), which showed that it is a complex phenomenon. Gene interactions at pollen sterility loci could produce partially sterile pollen. Neutral alleles for pollen fertility (Sn) that do not interact with typical indica (S) and japonica (S) alleles provide a platform to exploit the strong hybrid vigor derived from intersubspecific crosses by controlling the reproductive obstacles between japonica and indica hybrids. Substantial evidence has shown that neutral genes, Sa”, Sb”, Sc”, Sd”, and Se”, provide valuable gene resources to surmount the pollen sterility related to the respective locus [4–8].

Autotetraploid rice is another viable option for increasing the yield of rice. Autotetraploid rice has a double genome compared with diploid rice and has wide adaptability [9–12]. Intersubspecific autotetraploid rice hybrids have more significant biological and yield potential than diploid rice [13–16]. Our previous research had demonstrated that polyploidy increased chromosomal abnormalities and enhanced pollen sterility loci interactions that cause low pollen fertility in autotetraploid hybrids [17–21]. However, the autotetraploid rice hybrids, which harbored double-neutral genes, Sa” and Sb”, displayed high seed setting, high pollen fertility (>70%) and significant positive hybrid vigor for yield and yield-associated traits [22–24]. Cytological observations also showed a higher frequency of bivalents and normal chromosome behaviors in these hybrids than parents during meiosis. The double neutral genes, Sa” and Sb”, could overcome hybrid sterility in diploid and autotetraploid rice hybrids [7, 24].

In recent years, the transcriptome analysis has become particularly widespread. The rapid development of next-generation sequencing has opened exciting avenues for investigating gene expression and function. The RNA-seq has been employed to identify differentially expressed genes between autotetraploid and diploid rice hybrids that heterozygous (SS’S’) at Sa, Sb and Sc pollen sterility locus during pollen development. The results revealed that the polyploidy enhanced epistatic interactions between alleles of these three sterility loci, and the interaction at Sb was more robust than other loci [20, 25]. T449 is an important autotetraploid rice line, which contains double neutral genes, Sa” and Sb”. The genomic variants were detected by re-sequencing between diploid (E249) and autotetraploid rice line (T449), and polyploidy not only induced abrupt changes in the expression patterns of meiosis-related genes which resulted in the abnormal chromosome behavior but also caused variations in the expression levels of saccharide-related genes [23]. High pollen fertility was found in the hybrids developed by crossing T449 and near-isogenic lines. Several hybrid combinations were generated by crossing T449 with neo-tetraploid rice lines, and differentially expressed genes were detected between parents and hybrids in nine tissues at different development stages. The results showed significantly higher expression patterns of necessary starch synthase and saccharides
metabolism-related genes. Many meiosis-related and specific genes were found to be up-regulated in the hybrid compared to the parent with low seed set [23].

In this study, we developed autotetraploid rice hybrids by crossing T449 with E24-4x, which contained double neutral genes, \(S_a^n\) and \(S_b^n\), to evaluate the effect caused by the polyploidy, and its diploid counterpart was used as control. Moreover, chromosome behavior during PMC meiosis and morphological traits were investigated in the hybrids and parents with different ploidy levels. Transcriptome analysis of anther development was also executed during a single microspore stage and meiosis to understand the gene expression profiles in the autotetraploid rice hybrid. The functional analysis of these transcripts may offer valuable information to decipher the molecular mechanism underlying heterosis by using neutral genes for pollen sterility loci in autotetraploid rice hybrids.

**Materials and methods**

**Plant material**

A diploid rice cultivar, E249, and its autotetraploid rice line, T449, harboring \(S_a^n\) and \(S_b^n\) double neutral genes for pollen sterility loci, were used as maternal lines, and crossed with E24 and E24-4x to develop diploid rice hybrid (hereafter referred as DF\(_1\)) and autotetraploid rice hybrid (hereafter referred as AF\(_1\)). E249 (DN18) harbored \(S_a^n\) and \(S_b^n\) double neutral genes for pollen sterility loci, and E24 is a near-isogenic line of Taichung 65, which has the same genetic background as Taichung 65, except at \(S_a\), \(S_b\) and \(S_c\) pollen sterility loci. T449 and E24-4x were developed by our research group from diploid rice E249 and E24 through colchicine-mediated chromosome doubling and self-crossed for more than 25 generations, respectively. These materials were grown at the research station of South China Agricultural University (SCAU) under natural environment, and management practices were done according to the recommendations of the area.

**Cytological investigations**

The chromosome behaviors and configuration were investigated according to Chen et al. [23]. In short, the inflorescences of parental lines and \(F_1\) were fixed in Carnoy’s solution (ethanol: acetic acid = 3:1) for 24 h, and then kept at 4°C in 70% ethanol. After the removal of the floret, the anther was retained in a small drop of 1% acetocarmine on a microscope slide. After 2–3 min, a coverslip was used to cover the microscope slide and observed under a Motic BA200 microscope. The pollen fertility was detected by staining with 1% I\(_2\)-KI under a microscope (Motic BA200) [26].

**Evaluation of heterosis and agronomic traits**

The mid parent heterosis (MPH) and high parent heterosis (HPH) were evaluated by the following formula: MPH = (\(F_1\) − MP)/MP × 100%, and HPH = (\(F_1\) − HP)/HP × 100%, where MP represents the mean value of two parents, HP represents the value of best parent, and \(F_1\) indicates the performance of hybrid [12]. Agronomic traits, including filled grains per plant, 1000-grain weight, total grains per plant, effective number of panicles per plant, plant height, grain length and width, grain yield per plant, and seed set were investigated as described previously [12].

**RNA-seq and data analysis**

All the tissues of parents and hybrids were collected in three biological replicates and stored at -80°C for RNA isolation. The total RNA was isolated according to the manufacture’s protocol
of the TRIzol Reagent (Life Technologies, California, USA). The library was prepared according to the manual instructions as described previously [23, 24]. The low-quality data, including reads containing sequencing primer and nucleotides with quality score lower than 20, and sequencing adaptors, were discarded. All the data has been submitted to NCBI (Accession IDs: PRJNA656120, and transcriptome data of T449 and E249 was downloaded from NCBI under the accession number PRJNA436888 [23].

Differentially expressed genes in different samples were identified by Venny software (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Cluster 3.0 software was used for hierarchical clustering of all genes after normalization. For functional categorization, gene ontology (GO) analysis was conducted by using the agriGO v2.0 (http://systemsbiology.cau.edu.cn/agriGOv2/). Transcription factor (TF) analysis was performed based on transcription factor data [27].

Mapping of differentially expressed gene (DEG) to rice QTLs

The DEGs (DEGs between the parents and hybrid are labelled as DEGs\textsubscript{HP}) were mapped onto 26 yield-related traits and 1019 yield-related QTLs using gene coordinates from the MSU Rice Genome Annotation Project. Rice QTLs with physical locations on the MSU Rice Genome Annotation Project were downloaded from Gramene [28].

qRT-PCR validation

To validate the data of RNA-Seq by qRT-PCR, 10 DEGs were arbitrarily selected. Primer Premier 5.0 software was used to design gene-specific primers, and specific primers were examined in the NCBI database (S1 Table in S2 File). We take total RNA from sequenced samples, and the first-strand cDNA was produced using the Transcriptor cDNA Synthesis Kit (Roche) according to the manual instructions. The qRT-PCR reaction was performed on the Lightcycler480 system (Roche), and PCR profile was 30 s at 95˚C, with 40 cycles of 95˚C denaturation for 10 s and 60˚C annealing and elongation for 30 s. All qRT-PCR reactions were executed in three biological replications. The relative expression patterns of genes were estimated using the $2^{-\Delta\Delta Ct}$ method [29].

Results

Pollen fertility and chromosome behavior of hybrids and parents with different ploidy levels

In this study, T449 and E249, harboring $S_a^n$ and $S_b^n$ double neutral genes for pollen sterility loci, were used to develop autotetraploid rice hybrid (hereafter referred as AF\textsubscript{1}) and diploid rice hybrid (hereafter referred as DF\textsubscript{1}) by crossing with E24-4x and E24, respectively. There were different alleles at three pollen sterility loci (i.e. $S_a$, $S_b$, and $S_c$) in diploid and autotetraploid parents, so these hybrids presented allelic “interactions” at three pollen sterility loci. In diploid hybrid, pollen fertility was 68.67%, which was lower than its parents, and mid parent heterosis (MPH) and high parent heterosis (HPH) values for pollen fertility were -24.17% and -24.98%, respectively. However, pollen fertility was 76.74%, which was similar to their parents, and MPH and HPH values for pollen fertility were 4.43% and 1.59% in autotetraploid rice, respectively (Table 1; Fig 1). We detected high pollen fertility (>65%) in different ploidy hybrids with double neutral genes at $S_a$ and $S_b$ pollen sterility loci, and these results showed that different ploidy hybrids had no interaction at $S_a$ and $S_b$ loci. In addition, the MPH and HPH of autotetraploid hybrid for pollen fertility were higher than diploid hybrid, which exhibited that the effect of double neutral genes was stronger in autotetraploid hybrid.
Carmine acetate staining was employed to investigate the chromosome behavior in hybrids and parents with different ploidy (S1 and S2 Figs in S1 File). The chromosome behavior of diploid rice hybrid and its parents was similar, but in autotetraploid rice, the chromosomal abnormalities of AF1 hybrid was lower than its parents in the metaphase I, metaphase II, anaphase I and anaphase II (Fig 2; S2 Table in S2 File). These results indicated that autotetraploid hybrid chromosome behavior was better than parents.

The phenotype of parents and hybrids with different ploidy levels

The plant height (PH) was significantly higher in hybrids than parents (S3 Table in S2 File). For the effective number of panicles per plant (EP), 1000-grain weight (TGW), filled grains per plant (FG), grain yield per plant (GYP), seed set (SS), MPH and HPH were higher in autotetraploid hybrid than a diploid hybrid (S3 Table in S2 File; S3 Fig in S1 File). In particular, MPH and HPH for filled grains per plant (FG) were 149.22% and 62.21%, for grain yield per plant (GYP) were 196.68% and 158.33%, and for seed set (SS) were 214.68% and 158.33%, respectively. These results showed that heterosis was more substantial in autotetraploid hybrid than diploid counterpart.

Transcriptome profiles of rice anthers

To understand the gene expression profile in the autotetraploid rice hybrid, the RNA-seq experiments were conducted with different ploidy hybrids and their parents in anthers at

Table 1. Pollen fertility of hybrids and parents with different ploidy levels harboring genetic interactions at Sa, Sb and Sc loci.

| Material | Genotype at Sa, Sb and Sc pollen sterility loci | Pollen fertility (%) | MPH | HPH |
|----------|-----------------------------------------------|----------------------|-----|-----|
| E249     | nn/nn/ii/ii                                   | 90.58±1.79           | -24.17 | -24.98 |
| DF$_1$   | ni/ni/ij                                      | 69.44±2.88           | -       | -       |
| E24      | ii/ii/ii                                      | 92.56±1.22           | -       | -       |
| T449     | nnnn/nnnn/nnn/nnn/ijij                       | 75.03±2.26           | 4.43  | 1.59  |
| AF$_1$   | nnii/nnii/iiij                               | 76.22±3.82           | -       | -       |
| E24-4x   | iii/iii/iiii                                 | 70.94±0.97           | -       | -       |

E24 and E249 indicate diploid rice lines, and DF$_1$ indicates their F$_1$ hybrid; E24-4x and T449 indicate autotetraploid rice lines, and AF$_1$ indicates their F$_1$ hybrid.

https://doi.org/10.1371/journal.pone.0239377.t001

Fig 1. Pollen fertility of hybrids and parents with different ploidy levels. A, E249 (Diploid parent harboring double neutral genes); B, DF$_1$ (F$_1$ diploid hybrid); C, E24 (Diploid Parent); D, T449 (Autotetraploid parent harboring double neutral genes); E, AF$_1$ (F$_1$ Autotetraploid hybrid); F, E24-4x (Autotetraploid parent). Round and fully stained pollens represent fertile pollens (green arrows), while other represent abnormal pollens (white arrows). Bar = 100 μm.

https://doi.org/10.1371/journal.pone.0239377.g001
Fig 2. Frequency of normal cells in different ploidy hybrids during PMCs meiosis.

https://doi.org/10.1371/journal.pone.0239377.g002
meiotic and single microspore stages. At meiosis and single microspore stages, the raw reads of the 36 samples were ranged from 18 to 22 million clean reads in anthers (S4 Table in S2 File). The clean reads were mapped onto the Nipponbare reference genome, and 84.28% to 90.41% annotated transcripts of the reference genome was detected in our material (S4 Table in S2 File). We removed some of the weak correlation coefficient samples, and kept more than 0.8 correlation coefficient (S4 Fig in S1 File). In total, ten genes were randomly selected from RNA-seq data to validate by qRT-PCR, including MADS gene (MADS56, Os10g0536100), meiosis related-gene (Os03g0800200), F-box gene (Os06g0713400), 60S acidic ribosomal protein (Os12g0133050), glutathione S-transferase (Os01g0933900), signal peptidase homologue (Os05g0297900), two hypothetical proteins (Os12g0550600 and Os12g0550600) and two expressed proteins (Os03g0249700 and Os06g0574900). The qRT-PCR results were similar to the RNA-seq data (S5 Fig in S1 File), which demonstrated that the RNA-seq data is dependable.

Differentially expressed genes (DEGs) were identified by using the two filter conditions (i.e. fold change (FC) higher than or equal to 2 and false discovery rate (FDR) less than or equal to 0.05). Using these standards, we identified 168 to 6640 DEGs in autotetraploid and diploid rice at the meiosis stage, and 7449 and 1480 DEGs were identified in diploid and autotetraploid rice, respectively (Table 2). DEGs between the parents and hybrid are labelled as DEGs\textsubscript{HP}, and the DEGs between the parents are called as DEGs\textsubscript{PP}. In total, 3604 and 1696 DEGs\textsubscript{HP} were found in diploid and autotetraploid rice at the meiosis stage, and 5345 and 549 DEGs\textsubscript{HP} at the single microspore stage, respectively (Table 2). A total of 904 and 68 DEGs\textsubscript{HP} were specifically identified in autotetraploid hybrid at meiosis and single microspore stages (Table 2). We primarily concentrated on these DEGs\textsubscript{HP} to detect genes related to autotetraploid fertility and heterosis.

The GO analysis of 904 DEGs\textsubscript{HP} showed that 41 GO terms were significantly enriched at the meiosis stage. In the biological processes category, 33 GO terms, including cellular nitrogen compound metabolic process, carboxylic acid biosynthetic process, DNA-dependent and regulation of transcription, were significantly enriched. In molecular function, eight GO terms, such as electron carrier activity, transcription factor activity and iron ion binding, were significantly enriched (Fig 3). No significant GO term was detected in the single microspore stage.

KEGG pathway analysis showed that 137 of 904 DEGs were associated with 21 functional terms at the meiosis stage, and 7 of 68 DEGs were enriched in 5 functional terms at the single microspore stage (Fig 4). Those, as mentioned earlier, 137 and 7 DEGs were enriched in 75 and 8 subcategories, and 16 and 2 significant terms were detected at the meiosis and single microspore stage, respectively (Table 3).

### Association of DEGs\textsubscript{HP} with QTLs for yield-related traits

We mapped DEGs\textsubscript{HP} detected in autotetraploid hybrid, which were specifically expressed in autotetraploid at meiosis, to identify QTLs associated with grain yield in the rice genome.

| Development stage | Sample         | DEGs\textsubscript{HP} | DEGs\textsubscript{HP} | DEGs\textsubscript{HP} |
|-------------------|----------------|-------------------------|-------------------------|-------------------------|
|                   |                | M/F\textsubscript{T}   | F/F\textsubscript{T}   |                          |
| Meiosis stage     | diploid        | 3562                    | 2842                    | 1145                    | 3604                     |
|                   | autotetraploid | 2711                    | 1529                    | 256                     | 1696                     |
|                   | autotetraploid-specific | 904                   |                         |                          |
| Single microspore stage | diploid        | 6640                    | 859                     | 4848                    | 5345                     |
|                   | autotetraploid | 1436                    | 395                     | 168                     | 549                      |
|                   | autotetraploid-specific | 68                     |                         |                          |
A large number of QTLs for yield-related traits were detected, including grain weight per plant, pollen fertility, spikelet number, 1000-grain weight and male fertility restoration. A total of 22 genes were mapped in the interval of 12 yield-related QTLs, including 1 grain number QTL, 1 pollen fertility QTL, 1 spikelet number QTL, 5 1000-grain weight QTLs and 4 male fertility restoration QTLs. Furthermore, 3, 2, 1, 9 and 7 genes were identified in the grain yield per panicle QTLs, pollen fertility QTLs, spikelet number QTLs, 1000-grain weight QTLs and male fertility restoration QTLs, respectively (Table 4). These results showed that several differentially expressed genes were related to yield-related traits in autotetraploid hybrid.

The mode of inheritance for DEGs_{HP}

The 904 and 68 DEGs_{HP} were classified into 12 categories, which were classified into four major expression groups according to the method of Yoo et al. [30], including additive dominance, parental expression level dominance (high parental expression level dominance and low parental expression level dominance), transgressive down-regulation dominance, and transgressive up-regulation dominance. A total of 24, 107, 198, 549, 26 genes were detected in the additive dominance, high parental expression level dominance, low parental expression
level dominance, transgressive down-regulation dominance and transgressive up-regulation dominance at the meiosis stage, and 21, 37, 4, 2 and 4 genes were detected in the additive dominance, high parental expression level dominance, low parental expression level dominance, transgressive down-regulation dominance and transgressive up-regulation dominance at the single microspore stage, respectively (S6 Fig in S1 File). Among the DEGs HP, 2.65% and 30.88% had additive dominance, 11.8% and 55.88% had high parental expression level dominance, and 11.8% and 55.88% had high parental expression level dominance.
dominance, 21.9% and 5.88% had low parental expression level dominance, 60.73% and 2.94% had transgressive down-regulation dominance, and 2.88% and 5.88% had transgressive up-regulation dominance at the meiosis and single microspore stage, respectively (Fig 5).

The higher-parent dominance and transgressive up-regulation dominance were considered autotetraploid potential heterosis genes to investigate heterosis related genes in autotetraploid rice hybrids. A total of 133 and 41 DEGs were detected in higher-parent dominance and transgressive up-regulation dominance at the meiosis and single microspore stage, respectively. Among these genes, six genes were found to be commonly expressed in both phases, including one gene encoded beta-amylase (Os03g0141200), two new genes (Oryza_newgene_118 and Oryza_newgene_309) and three genes encoding expressed protein (Os01g0521200, Os01g0612350, Os01g0612350).

Table 3. Significant KO terms of DEGs during meiosis (MA) and single microspore stage (SCP).

| Term Name | Description | Number of DEGs | p-value |
|-----------|-------------|----------------|---------|
| MA        | Glyoxylate and dicarboxylate metabolism | 17 | 3.26E-11 |
| ko00630   | Glyoxylate and dicarboxylate metabolism | 17 | 3.26E-11 |
| ko00260   | Glycine, serine and threonine metabolism | 10 | 6.67E-05 |
| ko00280   | Valine, leucine and isoleucine degradation | 7 | 2.25E-04 |
| ko00250   | Alanine, aspartate and glutamate metabolism | 7 | 5.17E-04 |
| ko00195   | Photosynthesis | 7 | 0.001053788 |
| ko00710   | Carbon fixation in photosynthetic organisms | 8 | 0.002301607 |
| ko00910   | Nitrogen metabolism | 5 | 0.003345682 |
| ko00220   | Arginine biosynthesis | 5 | 0.003345682 |
| ko00196   | Photosynthesis—antenna proteins | 3 | 0.008877552 |
| ko00592   | alpha-Linolenic acid metabolism | 5 | 0.009946881 |
| ko04146   | Peroxisome | 7 | 0.015584713 |
| ko00941   | Flavonoid biosynthesis | 4 | 0.017082881 |
| ko00480   | Glutathione metabolism | 7 | 0.018423848 |
| ko00640   | Propanoate metabolism | 3 | 0.022807086 |
| ko00053   | Ascorbate and aldarate metabolism | 4 | 0.03442308 |
| ko00270   | Cysteine and methionine metabolism | 7 | 0.036537999 |
| SCP       | Sulfur relay system | 1 | 0.020984252 |
| ko04122   | Folate biosynthesis | 1 | 0.038669226 |

Table 4. Significant DEGs mapped in each of the QTL regions.

| Trait name               | Chr | DEGs |
|--------------------------|-----|------|
| Grain yield per panicle  | 5   | Os05g0438500, Os05g0475400, Os05g0460000 |
| Pollen fertility         | 3   | Os03g0773800, Os03g0786100 |
| Spikelet number          | 5   | Os05g0223000 |
| 1000-grain weight        | 6   | Os06g0320500 |
| 1000-grain weight        | 1   | Os01g0860400, Os01g0869800 |
| 1000-grain weight        | 3   | Os03g0819600, Os03g0844700, Os03g0860100 |
| 1000-grain weight        | 7   | Os07g0170100, Os07g0152800 |
| 1000-grain weight        | 7   | Os07g0624900 |
| Male fertility restoration| 1   | Os01g0183400, Os01g0151200, Os01g0127900 |
| Male fertility restoration| 11  | Os11g0210600, Os11g0187500 |
| Male fertility restoration| 10  | Os10g0356000 |
| Male fertility restoration| 7   | Os07g0624900 |

https://doi.org/10.1371/journal.pone.0239377.t003

https://doi.org/10.1371/journal.pone.0239377.t004
We performed predicted protein-protein interaction analysis at the meiosis stage to detect interactions among these 133 genes, and three sub-networks were detected (Fig 6).

We compared the DEGs with the transcriptome data of Arabidopsis or rice meiosis stage-specific genes and meiosis-related genes [31–35], and detected one meiosis-related gene (Os01g0917500, MSP1) and two meiosis specific-genes (Os07g0624900 and Os04g0208600). Besides, one GA-stimulated transcript gene and three transcription factors were detected, including Os06g0266800, Os04g0574500 (GRF), Os03g0759700 (bHLH), and Os10g0531900 (bZIP).

**Discussion**

**The effect of double neutral genes is stronger in autotetraploid hybrid than diploid hybrid**

Autotetraploid rice hybrids between different subspecies (japonica and indica) have greater adaptability and higher yield potential than the diploid rice hybrid [13–15, 36, 37]. However, pollen abortion caused by multiple genes interaction, i.e. Sa, Sb and Sc pollen sterility loci, is vital factor that causes abortion of male meiocytes and low pollen fertility in autotetraploid and diploid rice hybrids. Polyploidy increased the multi-allelic interaction at three loci that enhanced chromosomal abnormalities compared to diploid rice [17, 18, 20, 25]. It had been
demonstrated that intersubspecific autotetraploid and diploid hybrid rice sterility could be overwhelmed by double neutral genes ($S_a^{nn}$ and $S_b^{nn}$) [7, 20]. All autotetraploid rice hybrids harboring these double neutral genes ($S_a^{nn}$ and $S_b^{nn}$) showed normal pollen fertility (>70%), some of them generated by crossing of neo-tetraploid rice with autotetraploid even exhibited high seed set and significant positive heterosis for yield and yield-related traits [22, 24]. Interestingly, the results of the present study indicated that the pollen fertility of autotetraploid hybrid with double neutral genes ($S_a^{nn}$ and $S_b^{nn}$) was similar to the parents. In contrast, pollen fertility in diploid hybrid was lower than its parents. These results were consistent with our previous study [25], who also detected high fertility in autotetraploid rice hybrids.

Analysis of the agronomic traits in both autotetraploid and diploid hybrids showed that the values for HPH and MPH of autotetraploid hybrid were positive for all the traits except grain length and grain width. In particular, the values of MPH were very high for FG and SS, which were 149.22% and 214.68%, respectively. The autotetraploid hybrid also showed positive and the highest HPH for these two traits, and the HPH values were 62.21% and 158.33%, respectively. However, MPH and HPH values for FG were 41.98% and -3.96%, and 5.61% and -4.35% for SS in diploid hybrid, respectively. These results indicated that autotetraploid hybrid displayed higher heterosis than diploid hybrid. Similarly, high heterosis values were detected in autotetraploid rice compared to diploid rice [14, 15, 37].

It is well known that meiosis is a critical process, and chromosome behavior had an essential impact on pollen fertility and seed setting in rice. Recent research showed that the frequency of normal chromosome behavior was higher in hybrids harboring $S_a^{nn}$ and $S_b^{nn}$ neutral genes than their parents [22, 24]. Here, the chromosomal abnormalities of autotetraploid
hybrid with double neutral genes was lower than its parents in metaphase I, metaphase II, anaphase I and anaphase II. But the frequency of chromosome behavior of the diploid rice hybrids carrying \( S_a^n \) and \( S_b^n \) neutral genes and its parents were similar. All these results demonstrated that neutral genes had a more significant impact on chromosome behavior in autotetraploid hybrids than diploid hybrids. Hence, we inferred that double neutral genes have stronger effect on autotetraploid rice than diploid rice.

Possible reasons for gene expression differences in different ploidy rice hybrids

Meiosis plays a crucial role in rice pollen development. Transcriptome analysis was most often used to explore the effect of interactions between pollen sterility loci and polyploidy on gene expression profiles in autotetraploid rice hybrids during PMC meiosis. In total, 55 meiosis stage-specific or meiosis-related genes increased pollen sterility loci interactions in autotetraploid rice hybrids [20]. The 26 meiosis-stage specific and four meiosis-related genes displayed up-regulation in autotetraploid hybrid harboring \( S_a^n \) and \( S_b^n \) neutral genes and paternal line compared to maternal line [24]. Here, 904 and 68 DEGs were specifically expressed in autotetraploid hybrid at meiosis and single microspore stages compared with diploid hybrid. The higher-parent dominance and transgressive up-regulation dominance genes were considered as autotetraploid potential heterosis genes, and 133 and 41 such genes were detected at the meiosis and single microspore stage, respectively. A meiosis-related gene (Os01g0917500, MSP1) and two meiosis-specific genes (Os07g0624900 and Os04g0208600) were identified. MSP1 gene encodes a Leu-rich repeat receptor-like protein kinase and is required to initiate anther wall formation in rice and to restrict the number of cells entering into female and male sporogenesis [38]. The meiosis specific-gene (Os07g0624900) encoded SKP1-like protein that is necessary for multiple cellular processes in eukaryotes, such as ethylene, jasmonate, gibberellin (GA), auxin and light responses [39]. ASK1 contributes to the regulation of synopsis and synaptonemal complex formation during early meiotic prophase, homolog juxtaposition, chromosome remodeling in Arabidopsis [40, 41]. LSK1, LSK2, LSK3 were specifically expressed in late pollen development stages and the elongating pollen tube in three Lily [42]. Another meiosis specific-gene (Os04g0208600) encoded F-box/FBD protein. The S-locus F-box protein is recognized as the pollen determining factor of S-RNase-based self-incompatibility in Rosaceae, Scrophulariaceae and Solanaceae [43]. In crops, some F-box genes are associated with flower development and flowering [44]. These results suggested that the expression profiles of these meiosis-specific or meiosis-related genes regulate the fertility of autotetraploid rice hybrid harboring double neutral genes.

Transcription factors (TFs) play vital roles in gene regulatory networks, and the interactions between TFs and their target genes regulate spatiotemporal gene expression levels. In the present research, three transcription factors were found to be encoded by the identified DEGs, including Os04g0574500 (GRF), Os03g0759700 (bHLH) and Os10g0531900 (bZIP). The GRF family plays a vital role in cell division and proliferation during leaf development in rice [45]. OsGRF12 (Os04g0574500) encodes a putative novel transcriptional regulator in rice, and its expression enhanced after treating with GA\(_3\) [46]. BHLH and bZIP transcription factors have been reported to involved in floral development, such as the pollen development and pollen fertility and floral transition and initiation [25, 47]. Signalling pathways, controlling many cellular processes, were mostly affected by TFs. However, there is little known about the genes regulated by TFs and their particular roles in rice plant metabolism. There seems to be several genes involved in the Leu-rich repeat receptor-like protein kinase gene, hormone-related genes (auxin transporter, gibberellin-regulated protein), F-box protein gene, GRF, bHLH,
bZIP transfactor family. These genes take part in many biological pathways, including cellular processes, transcription factors, signal transduction mechanisms and photosynthesis. These results would offer novel insights into the interaction network associated with male fertility in autotetraploid rice hybrid.

**The role of meiosis, photosynthesis and heterosis related genes in rice harboring double neutral genes**

Although heterosis has been widely investigated in rice breeding and plays a vital role in agriculture, our understanding of the molecular mechanism involved in heterosis is still weak. RNA-seq provides a very convenient platform to investigate the molecular basis of heterosis in rice. The high-throughput gene-expression profiling in heterotic cross combinations has been implemented to find a large number of DEG between hybrids and their parents [48]. Here, we revealed various genes associated with strong heterosis in autotetraploid hybrid by transcriptional profiling. For example, *OsAMTR1* (*Os05g0475400*), which is related to grain yield per panicle, encodes aminotransferase that have different isoforms expressing in mitochondria, cytosol and peroxisomes and associated with different cellular processes [49, 50]. *GLO1* (*Os03g0786100*), which is related to pollen fertility, encodes a protein with 369 amino acids, and is predominately expressed in rice leaves [51]. Glycolate oxidase (GLO) is a key enzyme in photorespiratory metabolism, and *PsbS1* (*Os01g0869800*) is related to 1000-grain weight, which encodes photosystem protein. The accumulation of this protein exerts control over photosynthesis in fluctuating light [52].

Functional analysis of hybrid transcriptomes indicated that these genes were involved in multiple metabolisms, which play crucial roles in meiosis and heterosis and participate in most vital metabolic pathways, such as amino acid metabolism, photosynthesis metabolism. Amino acid metabolism not only provides vital raw resources for synthesizing proteins, polypeptides and other nitrogen-containing substances but also provides the materials for the maintenance of life and normal metabolism. In this study, the analyzed data showed that 31 DEGs involved in the different amino acid metabolism were up-regulated at the meiosis. Many recent studies demonstrated that the balance of free amino acids are vital for the pollen fertility and anther development, and the concentrations of certain amino acids in maize leaves are strictly related to hybrid yield [53, 54].

The amino acids metabolism involved in this study included leucine, valine and isoleucine degradation, serine, glycine and threonine metabolism, aspartate, alanine and glutamate metabolism, cysteine and methionine metabolism. Serine metabolism is mostly related to photorespiration in plants where two molecules of glycine constitute and two other pathways of serine synthesis which represent the branches of glycolysis [55–57]. The glycerate and phosphorylated pathways of serine synthesis in plants are the important process linking carbon and nitrogen metabolism [58]. Complete oxidation of Valine, Leucine and Isoleucine effectively allows the formation of ATP in the mitochondria by oxidative phosphorylation. But it is mostly unknown about the metabolic pathways for these branched-chain amino acids breakdown so far in plants [59]. Glutamate is an active amino acid, which rapidly stimulated the expression of genes involved in signal transduction growth and transport [60]. Methionine, is a building block for protein synthesis, is the immediate precursor of S-adenosylmethionine which plays numerous roles in transmethylation reactions and the biosynthesis of polyamines and the phytohormone ethylene [61, 62].

Photosynthesis is an important biological process on this planet, which provides consumable energy for plant development. Heterosis is associated with increased photosynthesis. The photosynthesis process can be divided into biochemical and fluorescence processes, which is a
primary process converting CO₂ into organic compounds through solar energy [63]. Here, the DEGs_HT was significantly enriched in the photosynthesis metabolism process between autotetraploid hybrid and its parents. We found that 8 DEGs were involved in carbon fixation of photosynthesis (ko00710), including genes encoding photosystem II, ATPase. 7 and 3 DEGs were involved in photosynthesis (ko00195) and photosynthesis–antenna proteins (ko00196), including gene encoding chlorophyll A-B binding protein. GO annotation of cellular component showed that the DEGs significantly enriched in photosynthesis-related organelles. These results showed that the metabolic pathways of amino acids and photosynthesis have a more significant influence on the yield of autotetraploid rice hybrid compared to other metabolic pathways. The genes within yield QTLs involved in these two metabolic pathways are important candidate genes for heterosis and yield and probably associated with the high yield and heterosis in autotetraploid rice hybrid.

Supporting information

**S1 File.** S1 Fig Chromosome behaviors during PMC meiosis in DF₁ (diploid hybrid); S2 Fig Chromosome behaviors during PMC meiosis in AF₁ (autotetraploid hybrid); S3 Fig Morphological characteristics of F₁ hybrid and their parents with different ploidy levels. S4 Fig The correlation coefficient of different ploidy hybrids and their parents in anthers at meiosis and single microspore stages. S5 Fig Confirmation of the DEGs in T449 and autotetraploid F₁ hybrid during meiosis stage. S6 Fig Twelve possible additive and nonadditive gene expression patterns in autotetraploid hybrid relative to its parents.

(PDF)

**S2 File.** S1 Table. List of primers used for qRT-PCR. S2 Table. Number of observed cells and frequency of normal cells in different ploidy hybrids and parents. S3 Table Heterosis analysis of autotetraploid and diploid rice parents and F₁ hybrids. S4 Table The data of different ploidy hybrids and their parents in anthers at meiotic and single microspore stages.

(XLSX)

**Acknowledgments**

The authors thank Ms. Shuhong Yu and other lab members for assistance.

**Author Contributions**

**Conceptualization:** Lin Chen, Haibin Guo, Muhammad Qasim Shahid.

**Data curation:** Muhammad Qasim Shahid.

**Formal analysis:** Lin Chen, Haibin Guo, Shuling Chen, Huijing Yang, Fozia Ghouri, Muhammad Qasim Shahid.

**Funding acquisition:** Haibin Guo, Muhammad Qasim Shahid.

**Investigation:** Lin Chen, Haibin Guo, Shuling Chen.

**Methodology:** Lin Chen, Haibin Guo, Shuling Chen, Huijing Yang, Fozia Ghouri.

**Project administration:** Muhammad Qasim Shahid.

**Resources:** Muhammad Qasim Shahid.

**Supervision:** Muhammad Qasim Shahid.

**Validation:** Lin Chen, Haibin Guo, Huijing Yang, Fozia Ghouri.
Writing – original draft: Lin Chen, Haibin Guo, Shuling Chen, Huijing Yang, Fozia Ghouri, Muhammad Qasim Shahid.

Writing – review & editing: Lin Chen, Haibin Guo, Fozia Ghouri, Muhammad Qasim Shahid.

References
1. Kato S, Kosaka H, Hara S. On the affinity of rice varieties as shown by fertility of hybrid plants. Bull Sci Fac Agric Kyushu Univ. 1928; 3:132–147.

2. Ouyang Y, Zhang Q. Understanding reproductive isolation based on the rice model. Annu Rev Plant Biol. 2013; 64(1):111–135.

3. Ouyang Y. Progress of indica-japonica hybrid sterility and wide-compatibility in rice (in Chinese). Chin Sci Bull. 2016; 61:3833–3841.

4. Shi LG, Liu XD, Liu B, Zhao XJ, Wang L, Li JQ, et al. Identifying neutral allele Sb at pollen-sterility loci in cultivated rice with *Oryza rufipogon* origin. Chinese Science Bulletin. 2009; 54(20):3813–3821.

5. Liu B, Li JQ, Liu XD, Shahid MQ, Shi LG, Lu YG. Identification of neutral genes at pollen sterility loci Sd and Se of cultivated rice (*Oryza sativa*) with wild rice (*O. rufipogon*) origin. Genetics and Molecular Research. 2011; 10(4):3435–3445. https://doi.org/10.4238/2011.October.31.10 PMID: 22057998

6. Li JQ, Shahid MQ, Feng JH, Liu XD, Zhao XJ, Lu YG. Identification of neutral alleles at pollen sterility gene loci of cultivated rice (*Oryza sativa L.*) from wild rice (*O. rufipogon Griff.*). Plant Syst Evol. 2012; 298(1):33–42. https://doi.org/10.1007/s00606-011-0520-5.

7. Shahid MQ, Chen F, Li H, Wang S, Chen p, Lin SQ, et al. Double-neutral genes, *San* and *Sbn*, for pollen fertility in rice to overcome *indica* × *japonica* hybrid sterility. Crop Sci. 2013; 53(1):164–176.

8. Li HY, Wang SZ, Shahid MQ, Chen ZX, Wang L, Chen FY, et al. Excavation of neutral alleles *San*, *Sb*, and *Scn* from rice germplasm harboring *S* gene. Acta Agronomica Sinica 2013; 39 (8): 1366–1376.

9. Shahid MQ, Sun J, Wei C, Zhang P, Liu X. Studies on the abnormality of embryo sac and pollen fertility in autotetraploid rice during different growing seasons. Pak J Bot. 2010; 42(1):7–19.

10. Cai DT, Yuan LP, Lu XG. New strategy of rice breeding in the 21st century II. searching a new pathway of rice breeding by utilization of double heterosis of wide cross and polyploidization. Acta Agron Sin. 2011; 27:110–116.

11. Shahid MQ, Li YJ, Saleem MF, Naeem M, Wei CM, Liu XD. Yield and yield components in autotetraploid and diploid rice genotypes (*indica* and *japonica*) sown in early and late seasons. Aust J Crop Sci. 2013; 7(5):632–641.

12. Guo H, Mendrikahy JN, Xie L, Deng J, Lu Z, Wu J, et al. Transcriptome analysis of neo-tetraploid rice reveals specific differential gene expressions associated with fertility and heterosis. Sci Rep. 2017; 7:40139. https://doi.org/10.1038/srep40139 PMID: 28071676

13. Shahid MQ, Liu G, Li J, Naeem M, Liu X. Heterosis and gene action study of agronomic traits in diploid and autotetraploid rice. Acta Agr Scand B-Soil Plant Sci. 2011; 61(1):23–32.

14. Shahid MQ, Xu H, Lin S, Chen Z, Naeem M, Li Y, et al. Genetic analysis and hybrid vigor study of grain yield and other quantitative traits in autotetraploid rice. Pak J Bot. 2012; 44(1):237–246.

15. Wu JW, Hu CY, Shahid MQ, Guo HB, Zeng YX, Liu XD, et al. Analysis on genetic diversification and heterosis in autotetraploid rice. Springer Plus. 2013; 2(1):1–12. https://doi.org/10.1186/2193-1801-2-1 PMID: 23419944

16. Guo H, Shahid MQ, Zhao J, Li Y, Wang L, Liu X. Agronomic traits and cytogenetic evaluation of newly developed autotetraploid rice line. Pak J Agr Sci. 2016; 53(2):291–301.

17. He JH, Shahid MQ, Chen ZX, Chen XA, Liu XD, Lu YG. Abnormal PMC microtubule distribution pattern and chromosome behavior resulted in low pollen fertility of an intersubspecific autotetraploid rice hybrid. Plant Syst Evol. 2011; 291:257–265.

18. He JH, Shahid MQ, Li YJ, Guo HB, Cheng XA, Liu XD, et al. Allelic interaction of *F*1 pollen sterility loci and abnormal chromosome behaviour caused pollen sterility in intersubspecific autotetraploid rice hybrids. J Exp Bot. 2011; 62(13):4433–4445. https://doi.org/10.1093/jxb/er1096 PMID: 21624978

19. Wu J, Shahid MQ, Guo H, Yin W, Chen Z, Wang L, et al. Comparative cytological and transcriptomic analysis of pollen development in autotetraploid and diploid rice. Plant Reprod. 2014; 27:181–196. https://doi.org/10.1007/s00497-014-0250-2 PMID: 25262386

20. Wu J, Shahid MQ, Chen L, Chen Z, Wang L, Liu X, et al. Polyploidy enhances *F*1 pollen sterility loci interactions that increase meiosis abnormalities and pollen fertility in autotetraploid rice. Plant Physiol. 2015; 169(4):2700–2717. https://doi.org/10.1104/pp.15.00791 PMID: 26511913
21. Li X, Yu H, Jiao Y, Shahid MQ, Wu J, Liu X. Genome-wide analysis of DNA polymorphisms, the methylome and transcriptome revealed that multiple factors are associated with low pollen fertility in autotetraploid rice. PLoS One. 2018; 13(8):e0201854. https://doi.org/10.1371/journal.pone.0201854 PMID: 30080873

22. Wu J, Chen L, Shahid MQ, Chen M, Dong Q, Li J, et al. Pervasive interactions of Sa and Sb loci cause high pollen sterility and abrupt changes in gene expression during meiosis that could be overcome by double neutral genes in autotetraploid rice. Rice. 2017; 10:49. https://doi.org/10.1186/s12284-017-0188-8 PMID: 29197985

23. Chen L, Shahid MQ, Wu J, Chen Z, Wang L, Liu X. Cytological and transcriptome analyses reveal abrupt gene expression for meiosis and saccharide metabolisms that associated with pollen abortion in autotetraploid rice. Molecular genetics and genomics. 2018; 293(6): 1407–1420. https://doi.org/10.1007/s00438-018-1471-0 PMID: 29974305

24. Chen L, Yuan Y, Wu J, Chen Z, Wang L, Shahid MQ, et al. Carbohydrate metabolism and fertility related genes high expression levels promote heterosis in autotetraploid rice harboring double neutral genes. Rice. 2019; 12:34. https://doi.org/10.1186/s12284-019-0294-x PMID: 31076936

25. Wu J, Shahid MQ, Chen M, Li X, Li J, Xu X, et al. Cytological and transcriptome analysis reveal that interaction at Sb pollen sterility locus cause down-regulation of important meiosis-related genes associated with high pollen sterility in autotetraploid rice hybrids. Plant Physiology and Biochemistry. 2019; 141:73–82. https://doi.org/10.1016/j.plaphy.2019.05.019 PMID: 31132695

26. Ghouri F, Zhu JN, Yu H, Wu JW, Baloch FS, Liu XD, et al. Deciphering global DNA variations and embryo sac fertility in autotetraploid rice line. Turkish Journal of Agriculture and Forestry. 2019; 43(6): 554–568.

27. Jin J, Tian F, Yang D, Meng Y, Keng L, Luo J, et al. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. Nucleic Acids Res. 2017; 45:D1040–D1045. https://doi.org/10.1093/nar/gkx1148 PMID: 21056153

28. Youens-Clark K, Buckler E, Casstevens T, Chen C, Declercq G, Derwent P, et al. Gramene database in 2010: updates and extensions. Nucleic Acids Res. 2011; 39:D1085–D1094. https://doi.org/10.1093/nar/gkq1148 PMID: 21076153

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C (T)) method. Methods (Orlando). 2001; 25:402–408.

30. Yoo MJ, Szadkowski E, Wendel JF. Homoeolog expression bias and expression level dominance in allopolyploid cotton. Heredity. 2013; 110(2):171–180. https://doi.org/10.1038/hdy.2012.94 PMID: 23169665

31. Fujita M, Horiuchi Y, Ueda Y, Mizuta Y, Kubo T, Yano K, et al. Rice expression atlas in reproductive development. Plant Cell Physiol. 2010; 51(2):2060–2081.

32. Tang X, Zhang Z, Zhang W, Zhao X, Li X, Zhang D, et al. Global gene profiling of laser-capture d pollen mother cells indicates molecular pathways and gene subfamilies involved in rice meiosis. Plant Physiol. 2010; 154(4):1855–1870. https://doi.org/10.114pp:110.161661 PMID: 20959420

33. Deveshwar P, Bovill WD, Sharma R, Able JA, Kapoor S. Analysis of anther transcriptomes to identify genes contributing to meiosis and male gametophyte development in rice. BMC Plant Biol. 2011; 11:78. https://doi.org/10.1007/s12284-011-0097-6 PMID: 21554676

34. Yant L, Hollister JD, Wright KM, Arnold BJ, Higgins JD, Franklin FCH, et al. Meiotic adaptation to genome duplication in Arabidopsis arenosa. Curr Biol. 2013; 23(21):2151–2156 https://doi.org/10.1016/j.cub.2013.08.059 PMID: 24139735

35. Wright KM, Arnold B, Xue K, Surinova M, O’Connell J, Bomblies K. Selection on meiosis genes in diploid and tetraploid Arabidopsis arenosa. Mol Biol Evol 2015; 32(4):944–955. https://doi.org/10.1093/molbev/msu398 PMID: 25543117

36. Tu SB, Luan L, Liu YH, Long WB, Kong FL, He T, et al. Production and heterosis analysis of rice autotetraploid hybrids. Crop Sci. 2007; 47(6):2356–2363.

37. Ghaleb MAA, Li C, Shahid MQ, Yu H, Liang JH, Chen RX, et al. Heterosis analysis and underlying molecular regulatory mechanism in a wide-compatible neo-tetraploid rice line with long panicles. BMC Plant Biology. 2020; 20:83. https://doi.org/10.1186/s12870-020-2291-z PMID: 32065735

38. Nonomura K, Miyoshi K, Eiguchi M, Suzuki T, Miyao A, Hirochika H, et al. The MSP1 gene is necessary to restrict the number of cells entering into male and female sporogenesis and to initiate anther wall formation in rice. Plant Cell. 2013; 15(8):1728–1739.

39. Tao T, Zhou CJ, Wang Q, Chen XR, Sun Q, Zhao TY, et al. Rice black streaked dwarf virus P7-2 forms a SCF complex through binding to Oryza sativa SKP1-like proteins, and interacts with GID2 involved in the gibberellin pathway. PLoS One. 2017; 12(5):e0177518. https://doi.org/10.1371/journal.pone.0177518 PMID: 28494021
40. Yang L, Wu Y, Zhang M, Zhang J, Stewart JM, Xing C, et al. Transcriptome, cytological and biochemical analysis of cytoplasmic male sterility and maintainer line in CMS-D8 cotton. Plant Mol. Biol. 2018; 97: 537–551. https://doi.org/10.1007/s11103-018-0757-2 PMID: 30066309

41. Zhao D, Yang X, Quan L, Timofejeva L, Rigel NW, Ma H, et al. ASK1, a SKP1 homolog, is required for nuclear reorganization, presynaptic homolog juxtaposition and the proper distribution of cohesins during meiosis in Arabidopsis. Plant Molecular Biology. 2006; 62:99–110. https://doi.org/10.1007/s11103-006-9006-1 PMID: 16897472

42. Chang LC, Guo CL, Lin YS, Fu H, Wang CS, Jauh GY. Pollen-specific SKP1-like proteins are components of functional SCF complexes and essential for Lily pollen tube elongation. Plant & cell physiology. 2009, 50(8): 1558–1572.

43. Huang J, Zhao L, Yang Q, Xue Y. AhSSK1, a novel SKP1-like protein that interacts with the S-locus F-box protein SLF. The Plant Journal. 2006; 46(5):780–793. https://doi.org/10.1111/j.1365-313X.2006.02735.x PMID: 16709194

44. Terefe D, Tatlıoğlu T. Isolation of a partial sequence of a putative nucleotide sugar epimerase, which may involve in starch development in Cucumis sativus L.). Theor Appl Genet. 2005; 111:1300–1307. https://doi.org/10.1007/s00122-005-0058-4 PMID: 16133308

45. Fang R, Li L, Li J. Spatial and temporal expression modes of MicroRNAs in an elite rice hybrid and its parental lines. Planta. 2013; 238:259–269. https://doi.org/10.1007/s00425-013-1881-5 PMID: 23640684

46. Choi D, Kim JH, Kende H. Whole Genome Analysis of the OsGRF Gene Family Encoding Plant-Specific Putative Transcription Activators in Rice (Oryza sativa L.). Plant & Cell Physiology. 2004; 45(7): 897–904.

47. Wang Z, Cheng K, Wan L, Yan L, Jiang H, Liu, et al. Genome-wide analysis of the basic leucine zipper (bZIP) transcription factor gene family in six legume genomes. BMC Genomics. 2015; 16:1053. https://doi.org/10.1186/s12864-015-2258-x PMID: 26651343

48. Li D, Huang Z, Song S, Xin Y, Mao D, Lv Q, et al. Integrated analysis of phenome, genome, and transcriptome of hybrid rice uncovered multiple heterosis-related loci for yield increase. PNAS. 2016; 113(41): E6026–E6035. https://doi.org/10.1073/pnas.1610115113 PMID: 27663737

49. Liepman AH, Olsen LJ. Alanine aminotransferase homologs catalyze the glutamate: glyoxylate aminotransferase reaction in peroxisomes of Arabidopsis. Plant Physiol. 2003; 131:215–227. https://doi.org/10.1104/pp.011460 PMID: 12529529

50. Ricoult C, Echeverria LO, Cliquet JB, Limami AM. Characterization of alanine aminotransferase (AlaAT) multigene family and hypoxic response in young seedlings of the model legume Medicago truncatula. Exp Bot. 2006; 57(12):3079–3089. https://doi.org/10.1093/jxb/erl069.

51. Zhang Z, Lu Y, Zhai L, Deng R, Jiang J, Li Y, et al. Glycolate Oxidase Isozymes Are Coordinately Controlled by GLO1 and GLO4 in Rice. PLoS ONE. 2012; 7(6): e39658. https://doi.org/10.1371/journal.pone.0039658 PMID: 22761858

52. Hubbard S, Ajigboye OO, Horton P, Murchie EH. The photoprotective protein PsbS exerts control over CO2 assimilation rate in fluctuating light in rice. The Plant Journal. 2012; 71(3): 402–412. https://doi.org/10.1111/j.1365-313X.2012.04995.x PMID: 22413771

53. Tang H, Song Y, Guo J, Wang J, Zhang L, Niu N, et al. Physiological and metabolome changes during anther development in wheat (Triticum aestivum). Plant Physiology and Biochemistry. 2018; 132:18–32. https://doi.org/10.1016/j.plaphy.2018.08.024 PMID: 30172190

54. Römisch-Marg L, Spielbauer G, Schützenmeister A, Schwab W, Piepho HP, Genschel U, et al. Heterotic patterns of sugar and amino acid components in developing maize kernels. Theor Appl Genet. 2010; 120(2):369–381. https://doi.org/10.1007/s00122-009-1190-3 PMID: 19898829

55. Kiecłkowski LA, Givan CV, Hodgson JM, Randall DD. Subcellular location of NADPH-dependent hydroxypyruvate reductase activity in leaf protoplasts of Pisum sativum L. and its role in photorespiratory metabolism. Plant Physiol. 1988; 88(4):1182–1185. https://doi.org/10.1104/pp.88.4.1182 PMID: 18666441

56. Ros R, Cascales-Miriana B, Segura J, Anoman AD, Toujani W, Flores-Torner M, Rosa-Tellez S, et al. Serine biosynthesis by photorespiratory and non-photorespiratory pathways: an interesting interplay with unknown regulatory networks. Plant Biol. 2013; 15(4):707–712. https://doi.org/10.1111/j.1438-8677.2012.00682.x PMID: 23199004

57. Ros R, Muñoz-Bertomeu J, Krüeger S. Serine in plants: biosynthesis, metabolism, and functions. Trends Plant Sci. 2014; 19(9):564–569. https://doi.org/10.1016/j.tplants.2014.06.003 PMID: 24999240

58. Igamberdiev AU, Kieczkowski LA. The Glycerate and phosphorylated pathways of serine syntheses in plants: the branches of plant glycolysis linking carbon and nitrogen metabolism. Frontiers in Plant Science. 2018; 9:318. https://doi.org/10.3389/fpls.2018.00318 PMID: 29593770
59. Schertl P, Danne L, Braun HP. 3-Hydroxyisobutyrate dehydrogenase is involved in both, valine and isoleucine degradation in *Arabidopsis thaliana*. Plant Physiology. 2017; 175:51–61. https://doi.org/10.1104/pp.17.00649 PMID: 28705827

60. Kan CC, Chung TY, Wu HY, Juo YA, Hsieh MH. Exogenous glutamate rapidly induces the expression of genes involved in metabolism and defense responses in rice roots. BMC Genomics 2017; 18:186. https://doi.org/10.1186/s12864-017-3588-7 PMID: 28212609

61. Ravanel S, Gakière B, Job D, Douce R. The specific features of methionine biosynthesis and metabolism in plants. PNAS. 1998; 95(13):7805–7812. https://doi.org/10.1073/pnas.95.13.7805 PMID: 9636232

62. Cohen H, Salmon A, Tietel Z, Hacham Y, Amir R. The relative contribution of genes operating in the S-methylmethionine cycle to methionine metabolism in Arabidopsis seeds. Plant Cell Rep. 2017; 36:731–743. https://doi.org/10.1007/s00299-017-2124-1 PMID: 28289884

63. Rascher U, Nedbal L. Dynamics of photosynthesis in fluctuating light. Curr. Opin. Plant Biol. 2006; 9(6): 671–678. https://doi.org/10.1016/j.pbi.2006.09.012 PMID: 17011815