Identification and Verification of Genes Related to Pollen Development and Male Sterility Induced by High Temperature in Thermo-sensitive Genic Male Sterile Wheat Line YanZhan 4110S

Xuetong Yang
Northwest A&F University: Northwest Agriculture and Forestry University
Jiali Ye
Northwest A&F University: Northwest Agriculture and Forestry University
Fuqiang Niu
Northwest A&F University
Yi Feng
Northwest A&F University
Xiyue Song (songxiyue@nwafu.edu.cn)
Northwest Agriculture and Forestry University

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Abstract

Background: Environment-sensitive genic male sterility is of vital importance to hybrid vigor in crop production and breeding, therefore, it is meaningful to identify and study the function of the genes related to pollen development and male sterility, which still not fully understanding currently. In this study, Yanzhan 4110S, a new thermo-sensitive genic male sterility (TGMS) wheat line, and its near isogenic line Yanzhan 4110 were carried out cytological features observation, bioinformatics analysis to investgate the abortion state and identified the genes involved in pollen development which have fertility regulation function. Barely stripe mosaic virus-induced gene silencing was used to verify the genes function.

Results: Cytological analysis showed pollen abortion event of Yanzhan 4110S occur at the later uninucleate stage (Lun) under higher temperature induction (day/night temperatures of 22 °C/20 °C), when the anthers were collected and assessed for transcriptomic profiling through high-throughput sequencing. We then in-depth analyzed the differentially expressed genes (DEGs) by Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, the results showed that the occurrence of Yanzhan 4110S male-sterility most likely related to metabolic pathway, including phenylpropanoid biosynthesis in the biosynthesis of other secondary metabolites, starch and sucrose metabolism in carbohydrate metabolism, carbon fixation in photosynthetic organisms as well as carbon metabolism in energy metabolism. The weighted gene co-expression network analysis in the transcriptome profiles further identified some hub genes, where the key genes involved in those pathways were intersection between the unique DEGs of Yanzhan 4110S in anther and hub genes, totally 228 genes, which were highly related to pollen development including *TaMut11* and *TaSF3*. Moreover, further verification through barely stripe mosaic virus-induced gene silencing elucidated that the silencing of *TaMut11* and *TaSF3* caused pollen abortion, finally resulting in the declination of fertility. So, the genes *TaMut11* and *TaSF3* are related to fertility conversion of Yanzhan 4110S.

Conclusion: Through comparative transcriptome bioinformatics analysis, the genes *TaMut11* and *TaSF3* associated with pollen development and male sterility induced by high temperature were identified in Yanzhan 4110S, and verificated by barely stripe mosaic virus-induced gene silencing. These findings provided researching the abortive mechanism in environment-sensitive genic male sterility wheat.

Background

Wheat (*Triticum aestivum* L.), as one of the most widely cultivated crops, is vital food cereals worldwide [1]. With the emergence of an enormous population, insufficient cultivated lands, and the finite resources of reserve land, many efforts on sustainable development were made in increasing the cereals yield per unit area to satisfied the increasing global demand for cereals [2, 3]. The research shows that the utilization of crop heterosis is a very effective scientific way to increase crop yield, quality and breeding efficiency including wheat [4], rice [5], rape [6], maize [7], and soybean [8].

Male sterility is a main way to utilize heterosis [9]. Plant male sterility refers to the failure of the male organs in flowering plants to produce functional pollen, and the failure of anthers to crack leading to abortion, while the female gametes can develop normally, which simplify the procedure of hybrid in plant breeding [10]. There are various reasons for the occurrence of male sterility in plants, although it is the same crop, its classification also varies because of different basis. According to the causes of male sterility, it can be divided into genic male sterility (GMS), cytoplasmic male sterility (CMS), and environmental sensitive genic/cytoplasmic male sterility (EG/CMS). Where EG/CMS can be induced by external environment changes, such as temperature, light, nutrition, stress, and other external factors, and it is widely used in breeding system at present [11]. Wheat is a self-pollinated crop with small flower organs and difficult to remove stamens, male sterile lines as the female parent can improve seed production efficiency, ensure seed purity, reduce cost, and save labor. A series of male sterile wheat lines have been gradually selected and studied, but the molecular mechanism of its fertility conversion is unclear.

In recent years, with the development of high-throughput sequencing technology and the reduction of cost, sequence-based transcriptome analysis has become a comprehensive and accurate tool for determining gene expression patterns, which has the advantages of dynamic change, deep coverage and high resolution [12]. Transcriptome research is the basis and starting point of gene function and structure research. In a certain state, almost all transcriptional sequence information of specific tissues or organs of a species can be obtained by a new generation of high-throughput sequencing, which is convenient and fast. The available information includes gene expression, gene regulation, protein, and amino acid content [13]. As a good technology, high-throughput transcriptome sequencing has been paid more and more attention by researchers. Under the guidance of the rapid development of science and technology, male sterility research is also updated and improved. Transcriptome analysis can provide a new way to explore the mechanism of sterility [14], its application in the study of male sterility focuses on the differentially expressed genes (DEGs). It can compare the expression of a large number of genes in the same experiment, then analyze and study its internal mechanism through the method of bioinformatics, which is of great significance for the research in male-sterility [15], and we can better understand the genetic variation in the process of anther development and fertility change [16]. In recent years, RNA sequencing (RNA-Seq) has been applied for the key genes and vital metabolic pathway networks in a strong instrument. For example, soybean [17], rice [18], cotton [19], cabbage [20], wheat [21] and rice [22].

The way to identify the gene functions are usually gene silencing, complement function, and analyze mutant. Virus-induced gene silencing (VIGS) as a significant tool is used for study gene function in plants [23]. Compared with the traditional gene function research methods, it has the advantages of simple operation, low cost, fast obtaining phenotype, and no need to obtain stable strains. A few modified viral genomes were used for the purpose of VIGS vectors to target genes, which widely applied in plant development [24], plant nutrition [25], abiotic stress [26], disease resistance [27], the species involved wheat [28], soybean [29], barley [30], tomato [31], pea [32], and tobacco [33]. Barely stripe mosaic virus (BSMV), a positive sense RNA virus with a tripartite genome consisting of α, β, and γ, was applied to suppress genes successfully in plants [28, 34–36]. Especially, it is commonly applied in gene silencing

vectors in monocot species, especially wheat and barley [37, 38]. The BSMV virus system was successfully used in gene silencing for the first time in 2002. *PDS* gene was successfully inserted into the γ-RNA chain of BSMV by biotechnology, and infected barley leaves with reverse transcription system [30]. The results showed that the silencing of *TaPDS* gene in plants was induced, finally, BSMV induced barley endogenous gene silencing technology was successfully established and the BSMV was modified to improve the phenotypic effect of VIGS [39].

Yanzhan 4110S, a new thermo-sensitive wheat genic male sterility line, is completely sterility at high-temperatures (the daily average temperature above 20 °C at Zadoks growth stages 45–50) to produce hybrid seeds by cross pollination, and can self-pollinate at lower temperatures to maintain male-sterility, the fertility conversion was at the later uninucleate stage, which have the great potential for hybrid wheat breeding [40]. Althought the cause of its male-fertility conversion in cytology and metabolic pathway was previously reported [9, 40], the genes related to male sterility is still not confirmed clearly.

In our present study, we aim to identify the genes associated with male sterility in Yanzhan 4110S. Based on the core transcriptome sequencing data including near isogenic line Yanzhan 4110S and Yanzhan 4110 under different cultivated conditions, we have analyzed biological information including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Weighted Gene Co-expression Network Analysis (WGCNA) and carried out specific experiments, such as paraffin-cut section to observe the process of pollen development, BSMV-VIGS to identify the candidate genes function. We can provide fundamental in identifying great genes involved with fertility and further functional verification in the following research.

Results

Phenotyping for Yanzhan 4110S at different temperatures

The TGMS wheat line Yanzhan 4110S (abbreviated as AS) and its near-isogenic line Yanzhan 4110 (AF) used in this study were designated as ASd, ASg, AFd, and AFg according to the treated temperature (day/night temperatures of 17 °C/15 °C for ASd, AFd, and 22 °C/20 °C for ASg, AFg). To observe the development process of pollen grains by paraffin section, we divided the development process into tetrad stage (Td), late uninucleate stage (Lun), binucleate stage (Bn), and trinucleate stage (Tn). Compared with anthers of the other three samples (AFd, AFg, and ASd) under high and low temperature conditions, the microspore of cultured under high temperature (ASg) is not stained at Lun, and the pollen grains showed obvious shrinkage morphology, and the edge could not be stained in Tn (Fig. 1), which indicated that the pollen grains of ASg had an abortion eventually and led to male sterility. As expected for the previous result [40] showing the fertility of Yanzhan4110S can be regulated by temperature. Its anthers and pollens development have characterstics of sterility under high temperature conditions, while those of Yanzhang4110 were normal regardless of high or low temperature, and its fertility was sensitive to temperature.

Global Analysis Of Transcriptome Data

In total, we obtained 118.86 Gb clean data from the 12 libraries (AFg: T01, T02, T03; ASg: T04, T05, T06; AFd: T07, T08, T09; ASd: T10, T11, T12). The clean data of each sample reached 7.05 Gb, the percentage of Q30 was 92.20% or more, and the GC content was below 55.85%. The efficiency of the sequence alignment of clean reads from each sample compared with the wheat reference genome IWGSC_RefSeq_v1.0(https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0) ranged from 90.17–91.32% (Additional file 1: Table S1).

It is highly sensitive to detect gene expression by transcriptome data. In general, the FPKM values of protein-coding genes can be sequenced range from 10 ^ (− 2) to 10 ^ 4 [41]. The overall distribution of gene expression in samples was shown in Fig. 2a. which is normal with no bias found in the construction of cDNA library and gene expression level in each sample. The significance of the box plot is as follows: a) check the dispersion of gene expression level distribution of a single sample; b) visually compare the expression of genes in a variety of samples. In this study, the results reflected the differences in relative log RPKM values among 12 libraries were low (Fig. 2b). Pearson's correlation coefficient (r) is an index to evaluate the correlation of biological repetition [42]. The data were over 0.97 for all replicated samples which showed the consistent is strong for biological replicates (Fig. 2c). The PCA image also indicated that there was a great deal of correlation among the samples. The eigenvectors (57.5% and 23.3%) obviously separated all the sequencing samples (Fig. 2d). The above summary proved that the quality and accuracy of sequencing were reliable and can be for further study.

Differentially Expressed Genes (degs) Screening

For the samples with three biological duplications, software DEGseq [43] was used for the analysis of differential expression between sample groups and obtaining the differential expression gene set between two biological conditions. In the process of DEGs detection, ≥ 4-fold change and FDR < 0.01 were used as screening criteria. Overall, 2275 (1123 up-regulated, 1152 down-regulated), 3420 (2331 up-regulated, 1089 down-regulated), 4594 (3258 up-regulated, 1336 down-regulated) and 964 (290 up-regulated, 674 down-regulated) DEGs were obtained in the AFd Versus (VS) ASd (G1, T07_T08_T09 VS T10_T11_T12), ASd VS ASg (G2, T10_T11_T12 VS T04_T05_T06), AFd VS AFg (G3, T07_T08_T09 VS T01_T02_T03), and AFg VS ASg (G4, T01_T02_T03 VS T04_T05_T06), respectively (Fig. 3a-b). 1422 DEGs of which were the unique DEGs of AS, and were identified through Venn diagram (Fig. 3c).

Functional classification of DEGs by GO and KEGG pathway analysis

To further check the gene functional, the unique DEGs of AS that annotated were subjected to GO enrichment analysis. The 1422 DEGs were distributed over 43 significant functional groups in three broad categories of ontologies comprising “biological process” (BP), “molecular function” (MF) and “cellular component” (CC). In the BP, the DEGs were primarily concerned with the metabolic process (GO:0008152), cellular process (GO:0009987), single-organism process (GO:0044699), response to stimulus

(GO:0050896), and biological regulation (GO:0065007). The main MF contained 13 GO terms. Among these terms, catalytic activity (GO:0003824), binding (GO:0005488), and transporter activity (GO:0005215) were enriched to the high DEGs number. Cell part (GO:0044464), cell (GO:0005623), and organelle (GO:0043226) were dominant in the category of CC (Fig. 4).

KEGG pathways enrichment analysis was employed to study the major biological pathways where the 1422 DEGs participated. Five categories were identified including “metabolism”, “genetic information processing”, “cellular processes”, “organismal systems”, and “environmental information processing”. According to Global and overview maps, the most enrichment part was “metabolism” which composed of phenylpropanoid biosynthesis (belong to the biosynthesis of other secondary metabolites), starch and sucrose metabolism (carbohydrate metabolism), carbon fixation in photosynthetic organisms (energy metabolism), as well as carbon metabolism (energy metabolism) (Fig. 5). The results showed that the occurrence of AS male-sterility may be related to different genes and a complex pathways network.

Weighted Gene Co-expression Network Analyze Male-sterility Related Hub Degs

To furture explore target DEGs associated with male sterility in a network-level way, we constructed the weighted gene co-expression network analysis (WGCNA) with the 12 samples data, where the seed-setting rates were applied to analyze module-trait relationships. The result revealed that the module ‘Blue’ contained 1340 genes ($r = -0.73$, $p = 0.007$) was the most closely related to male sterility in the all samples (Fig. 6a-b). Furthermore, to further identify the molecular mechanism of male-sterility, we conducted KEGG enrichment analysis of the blue module. It mainly belonged to carbohydrate metabolism comprised of starch and sucrose metabolism and galactose metabolism, biosynthesis of other secondary metabolites including phenylpropanoid biosynthesis and flavonoid biosynthesis. Interestingly, many hub genes were consistent with the unique DEGs of AS mentioned above (Fig. 6c). Based on the results, we identified the candidate genes which were intersection between the unique DEGs of anther in AS and hub genes, totally 228 genes (Additional file 2: Table S2).

Functional Verification Of Candidate Genes Related To Fertility

Eight candidate genes that had highly relationships with male-sterility were selected for qRT-PCR analysis to confirm the real existence of gene. RNA samples from the anther of ASd, AFd, ASg, and AFg in Lun were used as templates. The results showed that the gene-expression patterns were the same between obtained by qRT-PCR and transcriptome sequencing, thus, the RNA-Seq results were precise and trustworthy, thereby demonstrating the associations among pathways, candidate genes and male fertility (Additional file 3: Fig. S1).

To further validate function, the two genes *TaSF3* and *TaMut11* related to pollen development were selected to carry out BSMV-VIGS experiment. In order to maintain gene silencing during anther development at Lun, the two infections were carried out, one week apart. The results showed that from 7 to 14 days of the first infection, the leaf phenotype of the plants inoculated with the virus was observed gradually. It was found that the leaf phenotype of uninfected plants was normal, the leaf color was dark green without any abnormality. The infection of BSMV: 000 resulted in streaks in the leaf, the plants inoculated with BSMV: *TaMut11* and BSMV: *TaSF3* showed obviously green fading and yellow stripe appearance, it was clear that infection was successful, and BSMV: *TaPDS* infected plants showed photobleaching and white spots, the results indicated *TaPDS* was silenced effectively that influenced the synthesis of carotenoids. (Fig. 7a). Moreover, the pollen grains present a sterile characteristics, brownish-yellow incomplete staining in the plants inoculated with BSMV: *TaMut11* and BSMV: *TaSF3*, and most of pollen grains can be stained normally in the plants inoculated with BSMV: 000, BSMV: *TaPDS* and control when the pollen grains in the middle of spikelets were stained with 1% KI-I_2 at the mature pollen grains stage (Fig. 7c).

In order to validate effectively silencing of genes,we firstly carried out qRT-PCR to determine the silencing effect of *TaPDS*, 7 to 14 days after the first infection, the leaves infected with BSMV:000, BSMV: *TaPDS*, and the control were collected when there are obvious bleaching spots exist in the leaves of the plant encroached BSMV: *TaPDS*. The expression of *TaPDS* gene in plants with infected BSMV: *TaPDS* were significantly reduced compared with that of control and BSMV:000 infected plants, which showed *TaPDS* gene was effectively silenced in the plants with BSMV: *TaPDS* infection (Fig. 8a). Subsequently, we detected the silencing effect of *TaMut11* and *TaSF3* genes that affects male fertility of Yangzhan 4110S by qRT-PCR, the anthers from the plants infected with BSMV:000, BSMV: *TaMut11*, and BSMV: *TaSF3* and control were as temples collected at Lun, the results showed that the expression levels of the two genes decreased significantly after silencing (Fig. 8b-c). As well as significantly decreased seed setting rates in the plants inoculated with BSMV: *TaMut11* and BSMV: *TaSF3* (Fig. 8d). Taken by these results we suggested that genes *TaMut11* and *TaSF3* relate to fertility conversion of Yanzhan 4110S.

Discussion

The determination of candidate genes related to male sterility

The application of heterosis is an essential approach in crop satisfying the global demand for food and improve the quality of wheat varieties at a high level [44]. Environmental sensitive male sterility is one of the major approaches to applying heterosis in wheat, the study of causes in the occurrence of male-sterility is valuable. In our study, the near isogenic lines Yanzhan 4110 (AF) and Yanzhan 4110S (AS) were used as experimental materials, and the two materials were treated with high temperature (22 °C/20 °C) and low temperature (17 °C/15 °C) in day/night, respectively. The dynamic development process of anther from Td to Tn was observed by paraffin section. The results showed that Yanzhan

4110S (ASg) was in an abnormal state under high-temperature treatment with the pollen grains shrank compared with other materials, eventually, resulting in male sterility. In order to identify the candidate genes that affect pollen development and male sterility, the high-throughput sequencing technology was employed to sequence the material AFd,ASd□AFg□ASg transcriptome. In our previous study, after high-temperature treatment, AS fertility directly changed to zero, and AF fertility also decreased due to the influence of high temperature, indicating that high temperature stress had a natural decrease in fertility of Yanzhan 4110 [9]. To eliminate the influence of environmental factors and material differences, we determined the DEGs of different combinations, including AFd_VS_AFg, AFd_VS_ASd, AFg_VS_ASg, and ASd_VS_ASg. The candidate genes were initially identified in the AS specific expression part of the four groups, including 1422 genes. Then, we carried out GO functional annotation and KEGG enrichment pathway analysis of these 1422 genes. The results showed that there are a lot of processes involved in the regulation of fertility change process, the “metabolism process” was the key part, including carbohydrate metabolism, energy metabolism, and biosynthesis of other secondary metabolites. In order to further accurately determine the candidate genes related to male sterility caused by abnormal pollen development in this experiment, AFd, ASd, AFg, ASg sequencing data were used to WGCNA analysis mapping the core genes related to fertility. The results showed that the blue module was the most closely related to fertility. Therefore, we once again made an intersection analysis between the genes in the blue module and the genes specifically expressed in anthers of the 1422 genes, and regarded the intersection genes as our final candidate genes, totally 228 genes, which further narrow the scope of screening candidate genes, the KEGG pathway and the genes are including carbohydrate transport and metabolism (beta-amylase and glycosyl hydrolases family 17); sulfur metabolism (Cytochrome c); lipid transport and metabolism (PI-PLC X domain-containing protein and GDSL-like Lipase/Acylhydrolase); starch and sucrose metabolism (pectinesterase, plant invertase/pectin methylesterase inhibitor); cell wall/membrane/envelope biogenesis (pectin lyase-like superfamily protein); signal transduction mechanisms (pollen-specific protein SF3, proline-rich receptor-like protein kinase, protein phosphatase 2C; protein phosphatase 2C and receptor-like protein kinase ANXUR1).

Genes TaMut11, **and** TaSF3 **related to pollen development**

In flowering plants, the stamen is the male reproductive organ which is composed of anthers and filaments. The process of pollen development is in the anthers, which is a precisely regulated process and the filament applies nutrients necessary for successful fertilization to the pollen development [45]. *LuWD40-1* encoding WD repeat protein has growth and pollen viability regulation ability in flax. The flax *LuWD40-1* mutant can not develop shoots. The pollen grains were empty, branches were reducing and flowering time was delayed leading to male sterile in overexpression lines [46]. In our study, the identified candidate *TaMut11* encoding WD domain/G-beta repeat protein which has a tight association with male sterility, and refers to many functional regulations including cellular process (GO:0009987), intracellular membrane-bounded organelle (GO:0043231), cytoplasmic part (GO:0044444), organic cyclic compound binding (GO:0097159) and heterocyclic compound binding (GO:1901363). *TaSF3* has LIM domain which refers to pollen development. The research showed *Arabidopsis* pollen-specific LIM proteins are involved in pollen development and tube growth. The *Arabidopsis PLIM2s* mutant completely destroyed the

process of pollen development, leading to pollen grains abortion and eventually male sterility [47]. There was a difference in the amount of actin protein between wild type fertile plants and male-sterile lines in the anther of wheat and Chinese cabbage, the anther in male-sterile lines has low actin levels, showing that the lower actin content associated with male sterility [48]. Above all, we chose *TaMut11*, *and TaSF3* as key genes related to male sterility for functional verification.

The fertility related genes can be effectively verified by BSMV-VIGS

Wheat is heterohexaploid crops including A, B, and D genome. Due to the genome is complex, the research of molecular mechanisms and genomics is difficult [49]. The genetic transformation of wheat is more difficult and time-consuming than other plants, therefore, VIGS is a good strategy for a fast functional study of genes in wheat BSMV belongs to the barley virus genus. After the virus infects the host plant, the host plant leaves will appear obvious dark green and light green or yellow infected spots in the early stage, and then gradually spread to the whole plant, and mechanical damage is one of the main ways of BSMV infection [50]. In recent years, especially in wheat and its related genera, a large number of genes related to stress resistance and disease resistance have been identified by BSMV-VIGS, such as *CYP96B22* [51], *TaEIL1* [52], *TaBTF3* [53], *MAPK* [54]. In this study, we used this technology to study the function of genes related to pollen development and proved *TaMut11* and *TaSF3* genes played an important role in the process of fertility. The wheat Yanzhan4110 were inoculated with BSMV: 000, BSMV: *TaMut11*, and BSMV: *TaSF3* and BSMV: *TaPDS.* The results showed the pollen grains of infected BSMV: *TaMut11* and BSMV: *TaSF3* plants had higher sterility rate in comparation with the contrast plants. The virus phenotype was observed in the leaves with green fading, the expression of the target genes were significantly decreased in the anther by qRT-PCR analysis, which indicating that the genes *TaMut11*, *and TaSF3* were effectively silenced by BSMV-VIGS system compared with the control plants. Therefore, *TaMut11* and *TaSF3* genes were down regulated by BSMV to achieve effective silencing, which affected the development of pollen grains in the growth process of plants, and thus could not complete the normal fertilization. The research provided a good foundation for further study of molecular mechanism in male sterility.

Conclusions

For EGMS line Yanzhan 4110S, the pollen grains were ultimately aborted under the high temperature condition according to the observation of the paraffin section. Based on the results, we further employed anthers RNA-Seq data to conduct bioinformatics analysis, which showed that there were 228 key genes in the set, the candidate genes *TaMut11* and *TaSF3* were screened and identified which closely related to pollen development. Moreover, further functional verification of the genes *TaMut11* and *TaSF3* was by BSMV-VIGS, due to the silencing of *TaMut11*, *TaSF3*, we found the genes expression were decrased significantly, the pollen grains were in sterility state through KI_2 dyeing, and the final seed set ratio were also in a significant downward trend, respectively. Therefore, the genes *TaMut11* and *TaSF3* have an important role in anther development, the silencing of them can lead to fertility decline which provide new insights into future studies in the mechanical involved with wheat pollen development in EGMS.

Materials And Methods

Plant materials and growth conditions

The TGMS wheat line Yanzhan 4110S (abbreviated as AS) and its near-isogenic line Yanzhan 4110 (abbreviated as AF) were used in this study. The seeds were planted in the pots during October 2017, at Northwest A&F University Experiment Station, Yangling, China (108°E, 34°15'N). At the connectives stage (Zadoks 37), the materials were moved to incubators with different temperatures. The treatments were designated as ASd, ASg, AFd, and AFg. The incubators were set with the intensity of 10000 lux and day/night period of 14 h/10h, ASd, AFd were at day/night temperatures of 17 °C/15 °C; and ASg, AFg were at 22 °C/20 °C. The anthers used for RNA-Seq at Lun were collected, frozen immediately in liquid nitrogen, and stored at – 80 °C with three biological replications each sample. We designed the number T01, T02, and T03 as AFg, T04, T05, and T06 as ASg, T07, T08, and T09 as AFd, T10, T11, and T12 as ASd. Moreover, anthers at the uninucleate (Un), Bn, and Tn were stored in formalin-acetic acid-alcohol (FAA) for cytological experiments, respectively.

Yanzhan 4110 were in the incubator at temperature of 25°C for BSMV-VIGS. The leaves were collected including control, BSMV: 000 (blank vector) and BSMV: *TaPDS* when bleached spots appeared on plants infected with BSMV: *TaPDS* to check the *TaPDS* silence situation, the anthers of control, BSMV: 000, BSMV: *TaMut11 and* BSMV: *TaSF3* at Lun were used to check the silence situation including *TaMut11 andTaSF3*. The spikes were bagged before anthesis, and seed setting rate, which is the percentage of seed number per spike to spikelet number, was calculated.

Paraffin section observations

Anthers at Td, Lun, Bn, and Tn were fixed in formalin-acetic acid-alcohol (FAA) at 4 °C. The samples were embedded in paraffin after dehydrating in ascending series of ethanol, cleared in xylene. The 5 μm thick sections were stained with toluidine blue and the images were captured by Axio Imager A2 (Germany).

RNA quantification and sequencing

The anthers collected at Lun in AFg, ASg, AFd, and ASd were used to construct cDNA libraries with 12 samples comprising T01, T02, T03, T04, T05, T06, T07, T08, T09, T10, T11, and T12 (3 biological replications of each material) for RNA-seq. The total RNA was isolated using the RNAprep Pure Plant Kit (TIANGEN BIOTECH (Beijing) Co. Ltd., China) follow the instructions. The concentrations were measured by a NanoDrop 2000 system (Thermo) and then the integrity was identified using an RNA Nano 6000 Assay Kit with an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The samples were processed by Biomarker Technologies Co, LTD (Beijing, China) and sequenced on an Illumina platform.

Data processing, identification and functional annotation of DEGs

Reads were filtered through quality check and high-quality clean reads (clean data) were identified for downstream mapping to wheat reference genome of (IWGSC_RefSeq_v1.0, https://urgi.versailles.inra.fr/

download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0) in HISAT2 v2.0.4 [55]. StringTie1.3.4b was used to assemble and quantify reads [56]. FPKM (Fragments Per Kilobase Million mapped reads) was estimated to check expression levels of genes, and the DEseq R package (1.10.1) [57] was used for differential gene expression analysis between samples. Genes with fold changes ≥ 4, FDR (False Discovery Rate) < 0.01 and P value < 0.05 were identified as DEGs. The DEGs were functionally annotated using BLAST2.2.31 [58] based on the GO (http://www.geneontology.org/) and KEGG (http://www.genome.jp/kegg/) databases.

Co-expression network construction

Weighted Gene Co-Expression Network Analysis (WGCNA) tool was conducted with all the genes to study the co-expressed gene modules highly relating to male-fertility. In order to identified modules with high specific expression, the correlation network of the WGCNA R package was applied for correlation analysis among highly correlated DEGs [59]. Module Eigengenes (ME) values were applied for estimating the association about modules and the male-fertility phenotype. The WGCNA network and ME were conducted by topological overlap matrices (TOM). The 1st principal element of a given module in ME was counted and used for evaluating the correlation between fertility and module.

Viral vector construction

In order to avoid functional redundancy and achieve complete silence, the cDNA fragment of each candidate (200-350bp) was applied for BSMV-VIGS experiment. The candidate genes *TaMut11 and TaSF3* were conducted by PCR amplification in Yanzhan4110 and the primers were shown in Additional file 4: Table S3. The vocters *α*、*β*、*γ*、*γ-TaPDS* used in the experiment are preserved in our lab. The BSMV-*TaMut11* and BSMV-*TaSF3* vectors construction were performed using γ-*PDS*, which was generated by digesting γ-*TaPDS* with *Not*I + *Pac*I and substituting for the *TaPDS* insert with annealed oligonucleotides forming a characteristic *Sma*I site between the *Not*I and *Pac*I cloning sites.

Viral RNA transcription

The linearized plasmid including *α* (digested with *Mlu*I)、*β* (digested with *Spe*I)、*γ* (digested with *Mlu*I), γ-*TaPDS*, γ-*TaMut11*,γ-*TaSF3* (all digested with *BssH*II) were used as template to conduct viral RNA transcription. The system is 20.0 μL including 5× T7 Transcription Buffer (4.0 μL), rATP (1.0 μL), rUTP (1.0 μL), rCTP (1.0 μL), rGTP (1.0 μL), 7mG (ppp) G RNA Cap (1.0 μL), T7 RNA Ploymerase (20 U μL^{-1}) (2 μL), RNase Inhibitor (40 U μL^{-1}) (1 μL), and linearized plasmid (α/ β/ γ/ γ-*TaPDS*/ γ-*TaMut11*/ γ-*TaSF3*) (8 μL), and bath at 37 ℃ for 1-2 hours to complete transcription.

Viral inoculation of BSMV-VIGS vectors

The same amount of in vitro transcripts including *α*/ *β*/ *γ*, *α*/ *β*/ *γ-TaPDS*, *α*/ *β*/ *TaMut11*, *α*/ *β*/ *γ-TaSF3* were mixed and diluted 5 times with FED solution. When the flag leaves are fully grown, BSMV inoculation was performed in the flag leaf and next leaf of wheat plants with a 10.5 μL transcript mixture

by friction method, and the leaves were infected again after a week to ensure *TaMut11* and *TaSF3* genes silencing at Lun. Six plants were infected with each of the candidate gene for qRT-PCR and seed set ratio calculation, three plants have the inoculation with BSMV: 000 and BSMV: *TaPDS* as control, respectively.

Quantitative real-time PCR validation

Eight genes were selected for qRT-PCR from the DEGs to confirm the existence of these genes, the total RNA were used consistent with the transcriptome sequencing samples. And transcription levels of genes, including *TaPDS,TaMut11,* and *TaSF3*, were measured also using the qRT-PCR to check the slience effect. The total RNA were extracted including a) the leaves of plants inoculated with BSMV: 000, BSMV: *TaPDS* and control when the bleached spots appeared to check *TaPDS* silence effect, b) the anthers of plants inoculated with BSMV: 000, BSMV: *TaMut11*, BSMV: *TaSF3*, and control at the later uninucleate stage to check *TaMut11* and *TaSF3* silence effect. The first-strand cDNA was synthesized by Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The primers were designed using Primer Premier 5.0 (Primer, Palo Alto, CA, USA) for qRT-PCR, and they were listed in Additional file 5: Table S4 and synthesised by TsingKe Biological Technology Co. Ltd (Xi'an, China). The qRT-PCR was performed in the QuantStudio™ 7 Flex Real Time PCR System (Applied Biosystems, USA). Three biological replicates were done for each material, the method was used according to Yang [9] and the relative gene expression levels were counted with the $2^{-\Delta\Delta CT}$ method [42].

Phenotypic characteristics

Anthers were used to determine fertility at the trinucleate stage by I_2-KI solution staining, images were captured using an Axio Imager A2 microscope. The images of wheat leaves and spikes were obtained using Olympus SZX10 (Japan). And the spikes were bagged before anthesis to calculate the seed setting rates.

Abbreviations

EGMS
Environment-sensitive genic male sterility
GMS
Genic male sterility
CMS
Cytoplasmic male sterility
ECMS
Environmental sensitive cytoplasmic male sterility
DEGs
Differentially expressed genes
RNA-Seq
RNA sequencing

VIGS
Virus-induced gene silencing
BSMV
Barely stripe mosaic virus
FAA
Formalin-acetic acid-alcohol
FPKM
Fragments Per Kilobase Million mapped reads
FDR
False Discovery Rate
WGCNA
Weighted Gene Co-Expression Network Analysis
ME
Module Eigengenes
qRT-PCR
Quantitative real-time PCR
Td
Tetrad stage
Lun
Late uninucleate stage
Bn
Binucleate stage
Tn
Trinucleate stage
AF
Yanzhan 4110
AS
Yanzhan 4110S
VS
Versus

Declarations

Ethics approval and consent to participate

This study does not contain any research requiring ethical consent of approval.

Consent for Publication

Not applicable.

Availability of data and material

All of datasets supporting the conclusions of this article are available in NCBI Sequence Read Archive under accession SRA: SRP211924.

Competing interests

The authors declare that they have no conflicts of interest

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Authors' contributions

XS and YF conceived and designed the study. XY performed the experiments, analyzed the data, and wrote the article with contributions from all of the authors. XS, JY, and FN revised and polished this manuscript. All authors have read and approved the manuscript.

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Figures

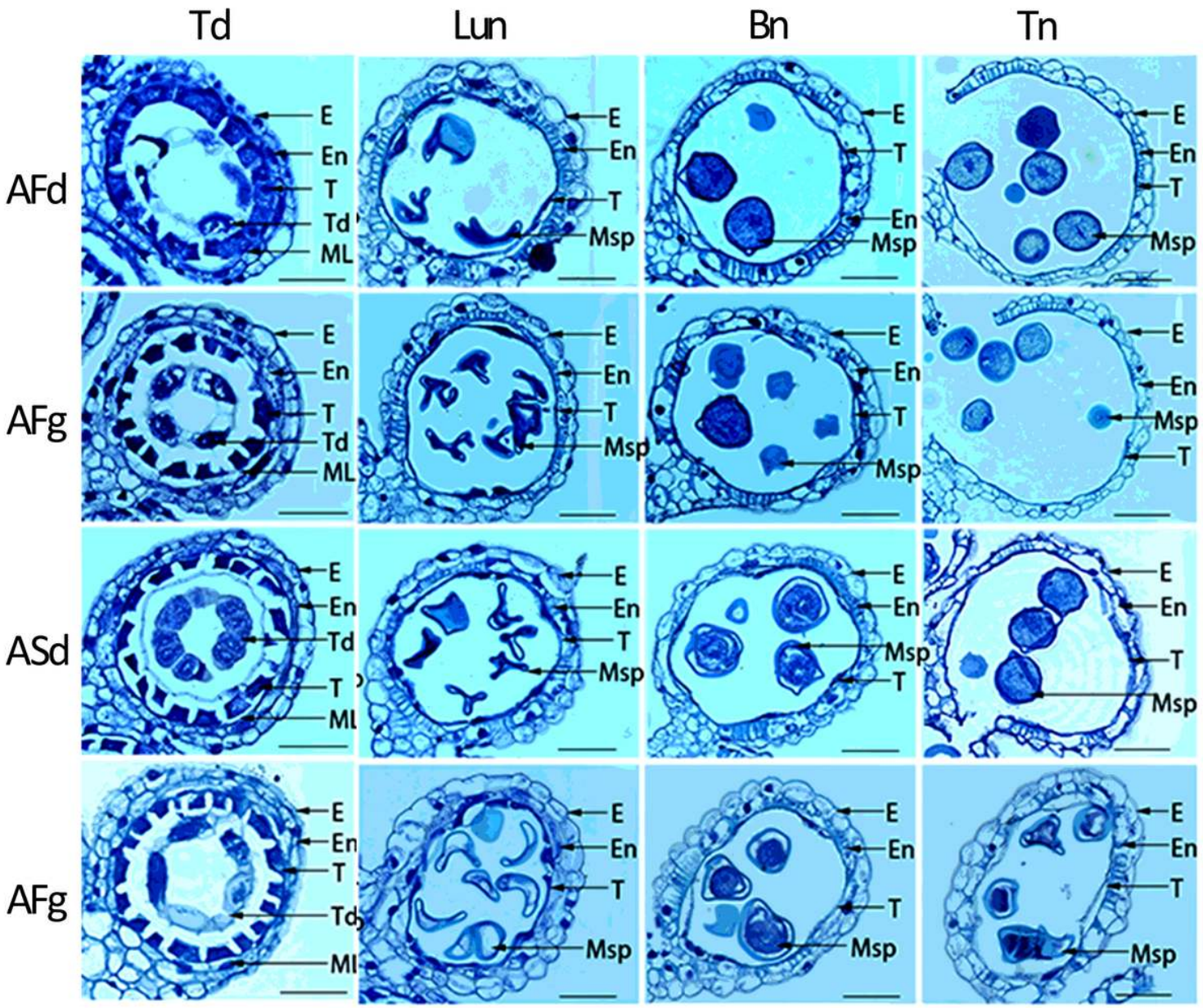

Figure 1

Comparison of anther paraffin section in wheat plants at different developmental stages. Td. tetrad stage; Lun. later uninucleate stage; Bn. binucleate stage; Tn. trinucleate stage; E. Epidermis; En. Endothecium; ML. Middle layer; T. Tapetum; Msp. Microspores Scale bars are 50 μm.

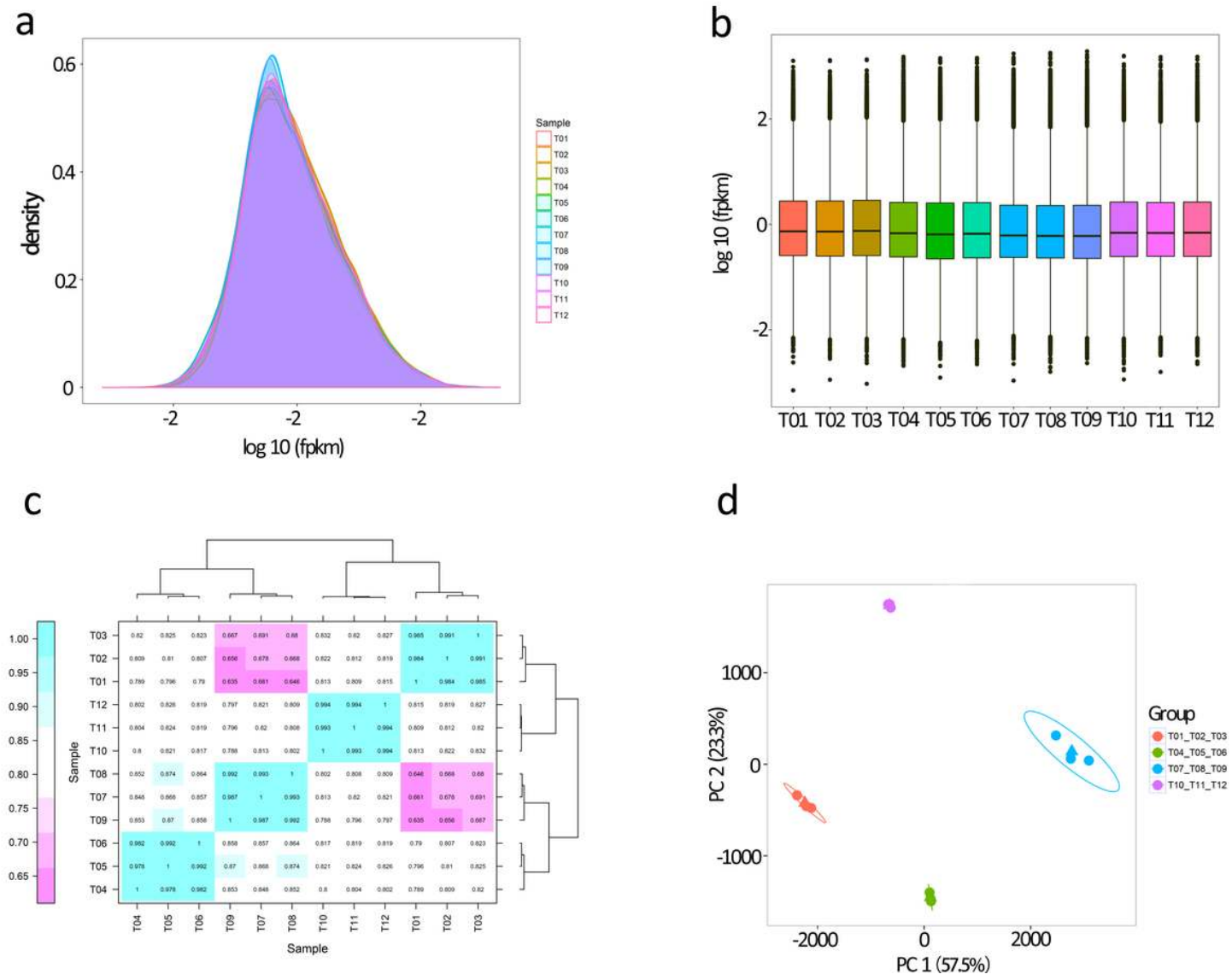

Figure 2

The Overview of Transcriptome Data. Comparison of FPKM density distribution (a). Box plot of relative log RPKM values of each sample (b). Pearson's Correlation Coefficient of the samples (c). PCA map of the samples (d).

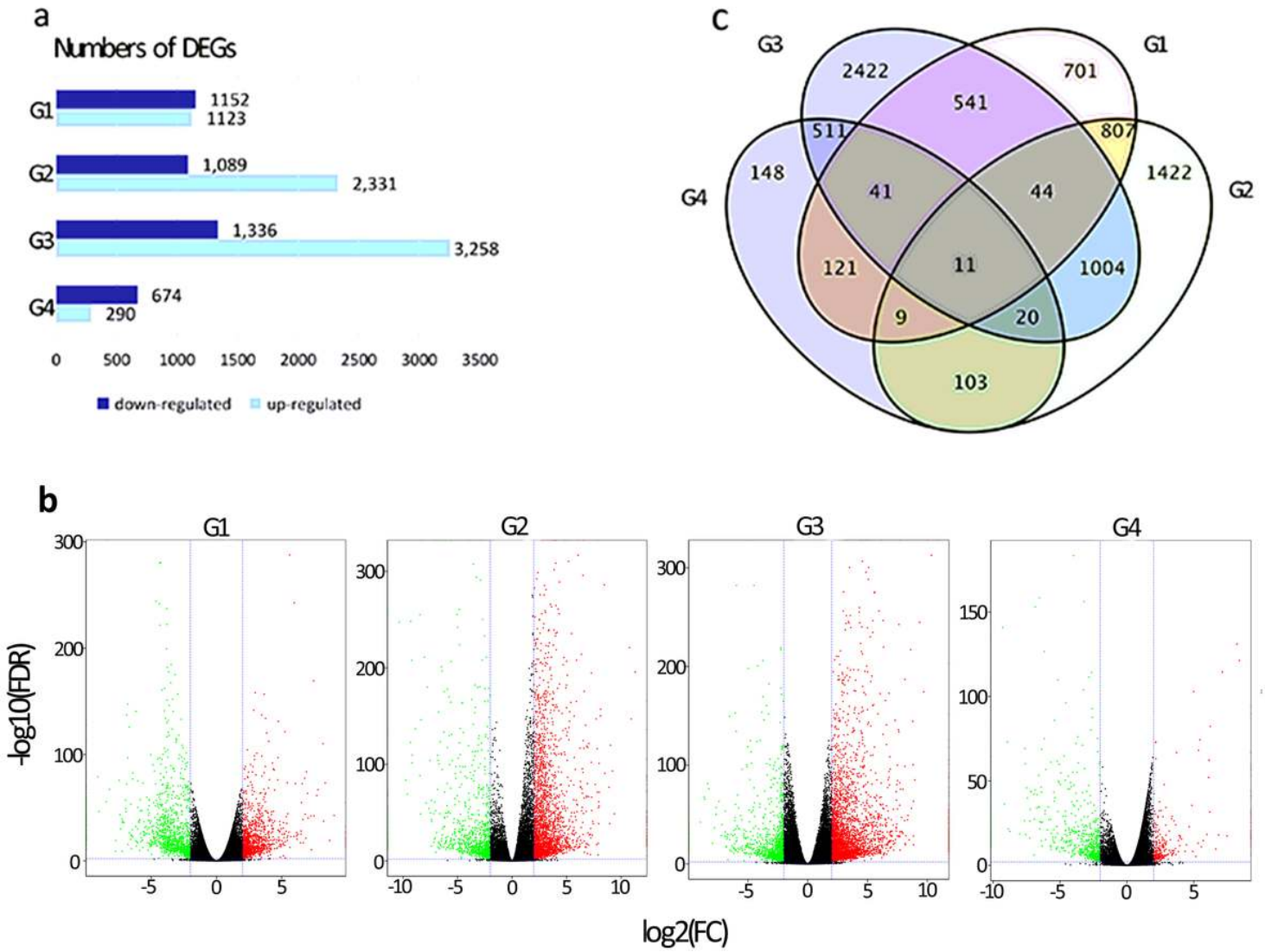

Figure 3

The number of DEGs between the samples. The total number of DEGs that up-regulated and down-regulated (a), Differences in the abundance of genes (b), Venn diagram of all DEGs (c).

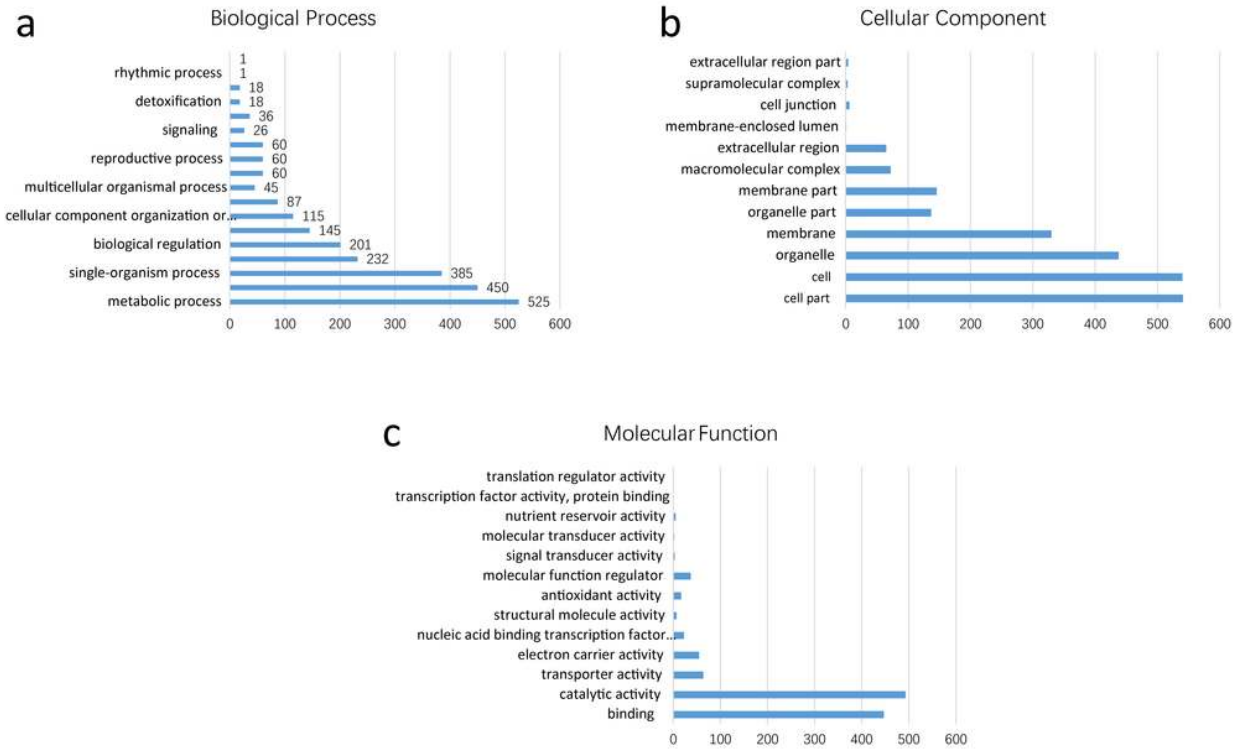

Figure 4

GO function enrichment of unique DEGs in Yanzhan 4110S. Biological processes (a). Molecular functions (b). Cellular components (c).

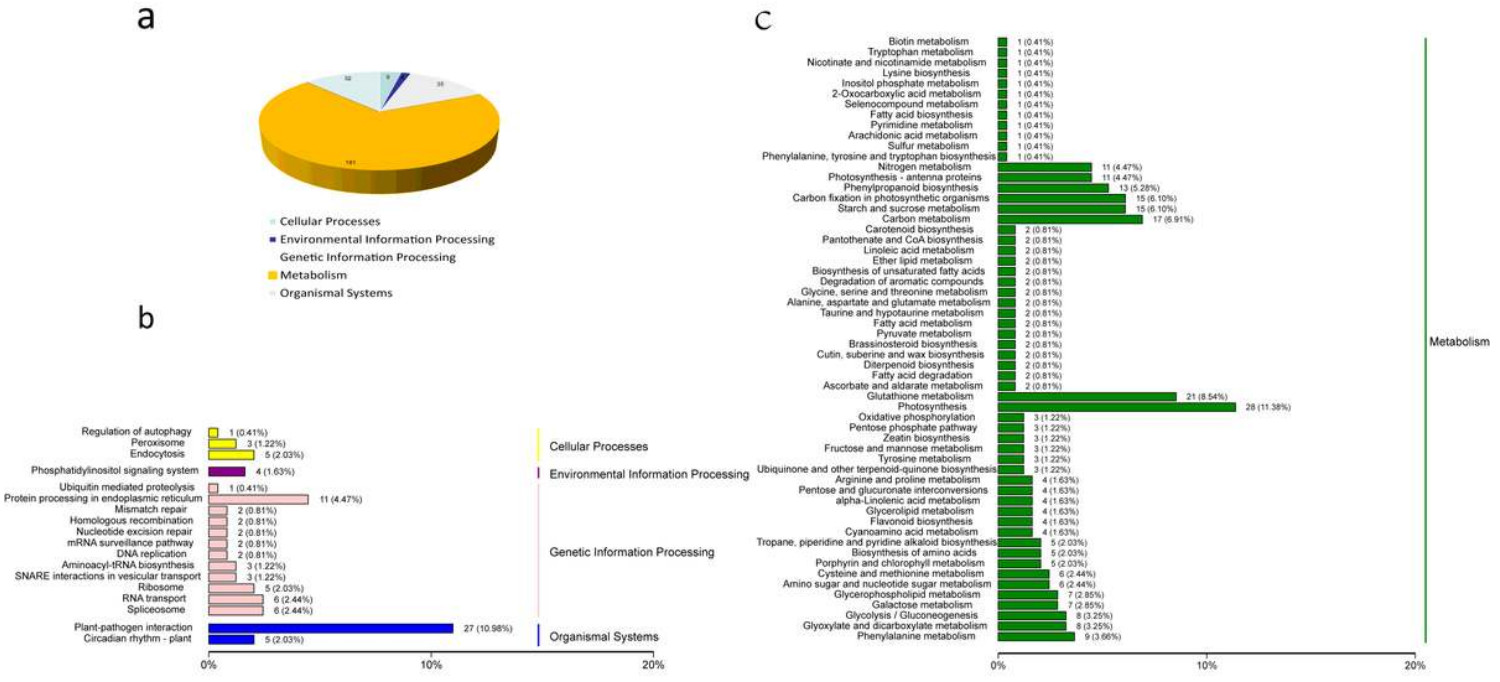

Figure 5

KEGG classifications of unique DEGs in Yanzhan 4110S.

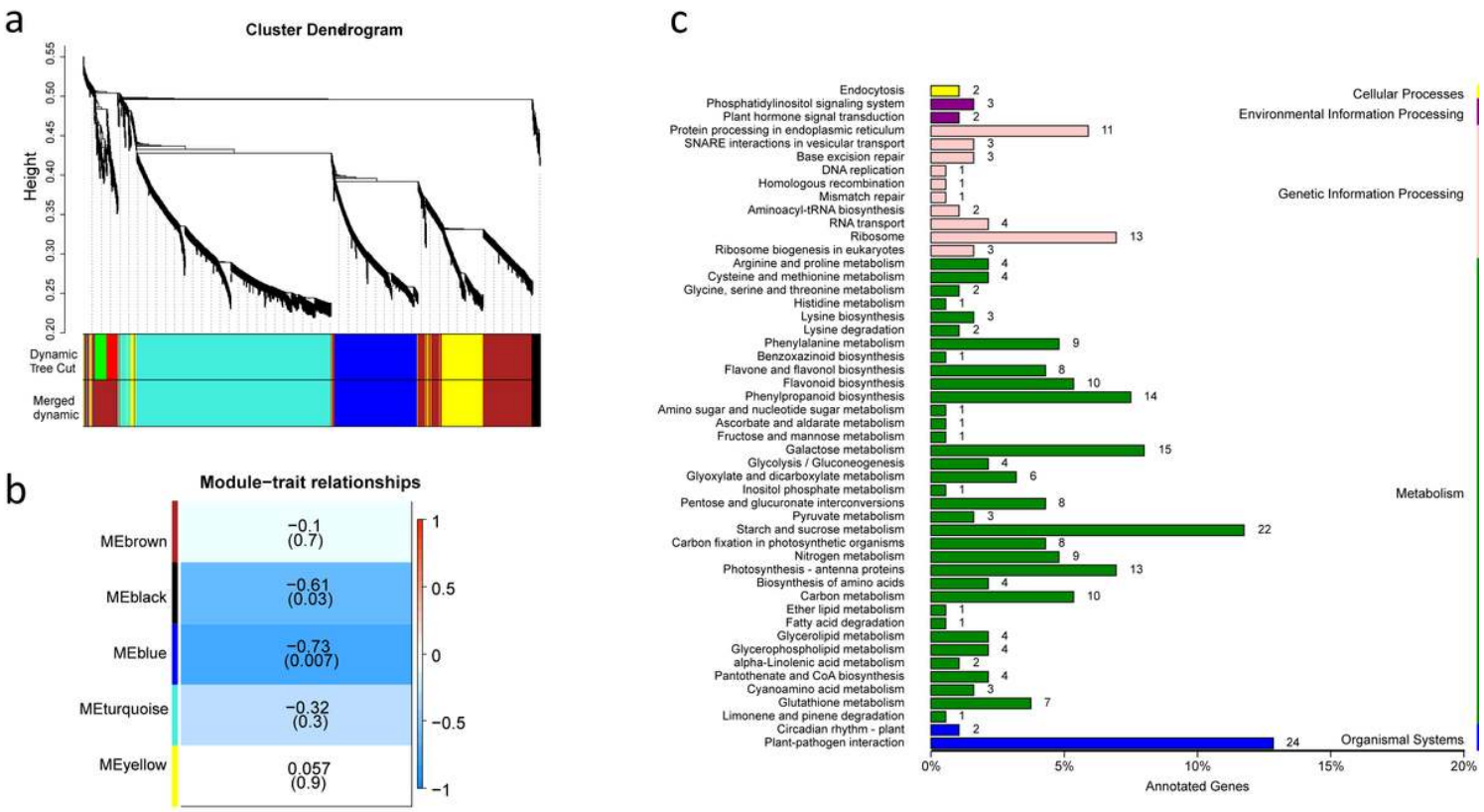

Figure 6

The co-expression modules of the male-sterility related genes analyzed via WGCNA. The co-expression modules of 12 sample genes (a). The module-trait correlation (b). The enriched KEGG pathways of the eigen genes in each module (c).

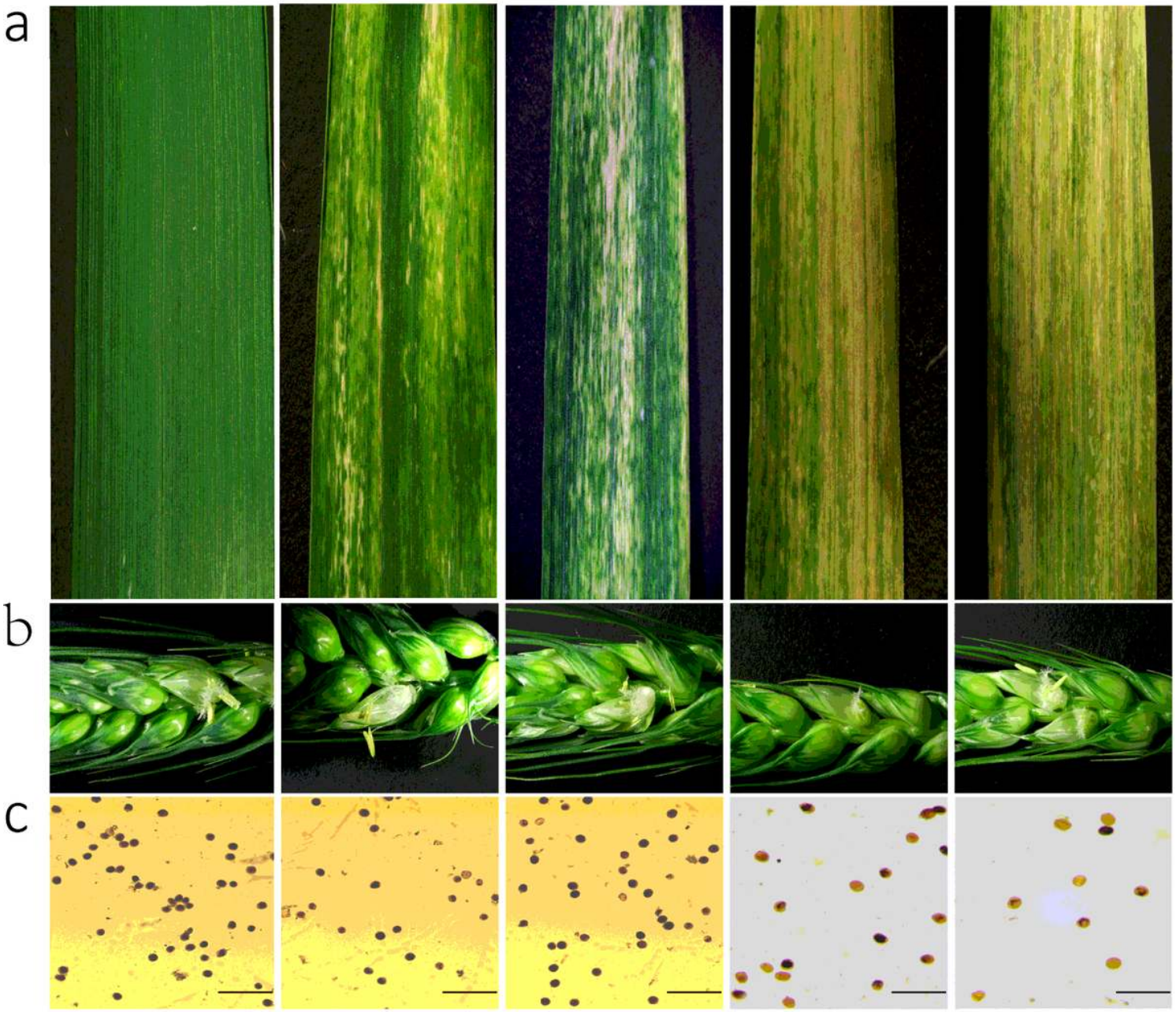

Figure 7

Phenotypic observation of leaves (a), spikelet (b) and I-KI2 staining (c). Each group order were contorl, Yanzhan4110 inoculated with BSMV:000, BSMV: TaPDS, BSMV: TaMut11 and BSMV: TaSF3, respectively. Scale bars are 50 μm.

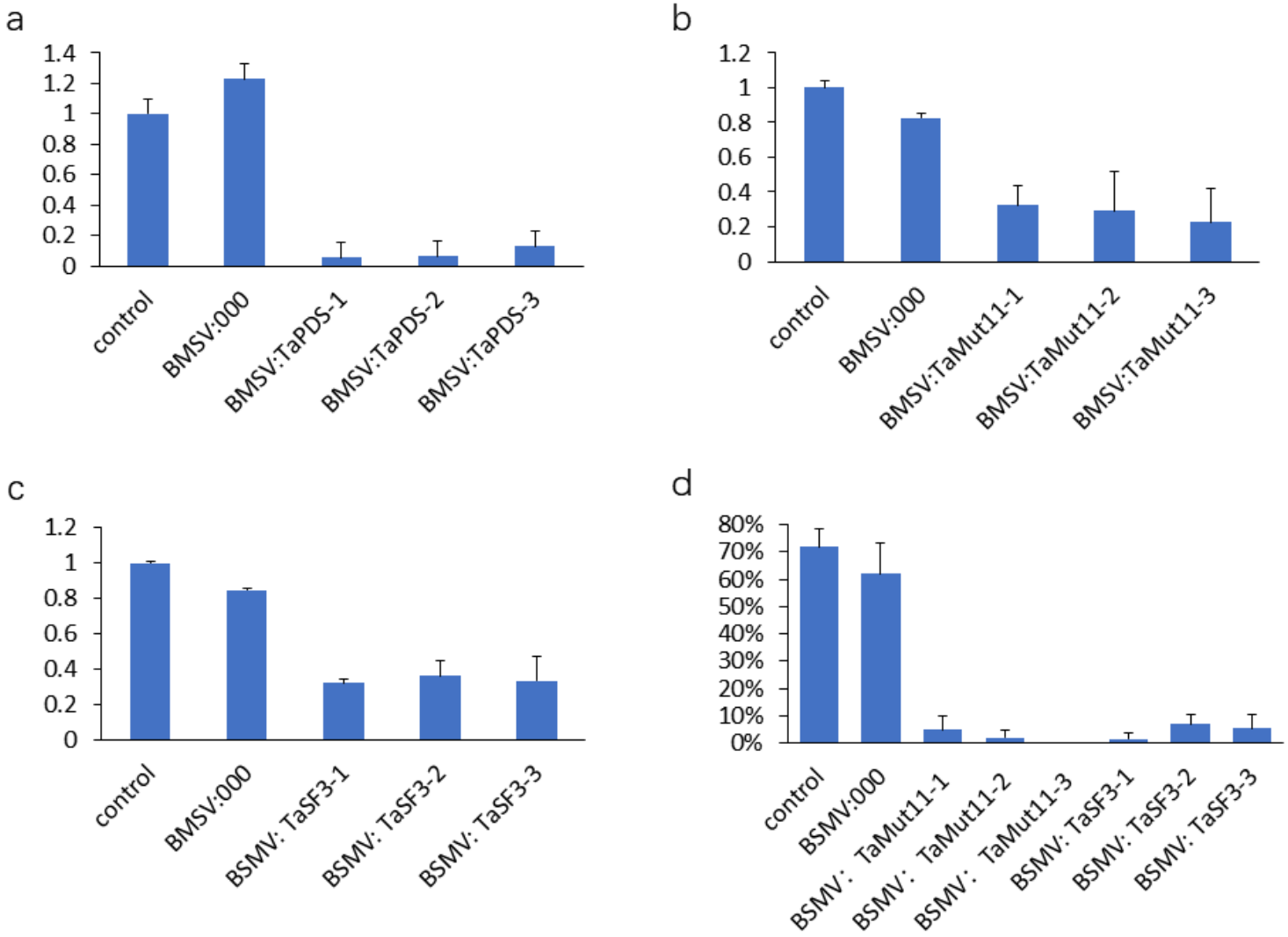

Figure 8

Sliecing effect of genes detected via RT-qPCR including TaPDS (a), TaMut11 (b) and TaSF3 (c). Seed set rates of plants inoculated with BSMV: 000, BSMV: TaMut11, and BSMV: TaSF3 and control (d). The data obtained represents three replicates and bars represent standard errors.

Supplementary Files

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