

# Comparative cytological and transcriptome analysis reveals high pollen fertility and upregulation of environmentally sensitive genic male sterility genes in neo-tetraploid rice

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## Original article

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# Abstract

**Background:** Autotetraploid rice is a useful germplasm for polyploid rice breeding; however, low seed setting is a major hindrance for the utilization of autotetraploid rice. Our previous study demonstrated that neo-tetraploid rice have great yield potential, which is thought to be one effective way to overcome the low fertility of autotetraploid rice. However, there is little known about the cause of high pollen fertility in neo-tetraploid rice. Here, we employed cytology and RNA-seq to study the molecular genetic mechanism of high pollen fertility in neo-tetraploid rice. **Results:** Cytological observations indicate that H1 displayed high pollen fertility (95.62%), lower percentage of pollen mother cells (PMCs) abnormalities, and stable chromosome configurations during the pollen development process compared with its two parents. RNA-seq analysis detected 1479 differentially expressed genes (DEGs) in neo-tetraploid rice compared with its two parents. Of these DEGs, 433 were annotated as pollen fertility-related genes, and 240 (~55.4%) exhibited significant upregulation in neo-tetraploid rice compared with its two parents, including nine cloned genes (TMS5, CSA etc.) that were validated by qRT-PCR and had been demonstrated to be pollen fertility-related genes. We further selected TMS5 as a candidate gene and analysed its phenotype in neo-tetraploid rice using the CRISPR/Cas9 technique. Significant variations have been detected in phenotypic charts, pollen development process and expression level in H1 and TMS5 knockout lines. **Conclusion:** Our finding provides strong evidence for the regulatory mechanisms of neo-tetraploid rice, and upregulation of pollen fertility-related genes should be associated with high fertility. Moreover, knockout of environmentally sensitive genic male sterility genes in the present study provide the new useful germplasm for polyploidy rice breeding.

## Background

Polyploidy is one of motivation in biological evolution, and it prevalently occurs in the plant evolution process (Doyle et al. 2008). Approximately 70% of plants have experienced at least one polyploidy during their evolutionary history (Masterson 1994). *Several advantages, including greater variation, high biomass yield and resistance to insect pest and diseases, were found in polyploidy species when compared with their original species* (Bingham et al. 1994; Marhold and Lihová, 2006). Two categories of polyploidy plants, *including the autopolyploidy and allopolyploidy species, usually exist in nature* (Comai, 2005). In contrast to the higher attraction of allopolyploidy plants, very few know the real appearance of autotetraploid plants in nature despite potential weaknesses, such as meiotic instability and reduced fertility. Increasing evidence indicates that the real appearance of autotetraploid plants in nature might be significantly underestimated (Parisod et al. 2010).

Autotetraploid rice is a useful germplasm derived from diploid rice by chromosome doubling. In comparison with corresponding rice, stronger biological vigour and heterosis were found in autotetraploid rice (Shahid et al. 2010; He et al. 2011; Wu et al. 2013); however, low pollen fertility is the major hindrance of its utilization (Wu et al. 2014; Chen et al. 2018). Pollen abnormalities appear to be the major obstacles for a normal seed set (He et al. 2011; Wu et al. 2014). Several previous studies have focused on the causes of low pollen fertility in autotetraploid rice, and these results were mainly focused on the abnormal pollen development process (He et al. 2011; Wu et al. 2014; Wu et al. 2015; Wu et al. 2017; Li et al. 2018; Wu et al. 2019). It is great demand to reveal the mechanism of its low pollen fertility and acquired the high pollen fertility tetraploid materials. After years of efforts, we successfully developed a new “autotetraploid rice lines” by selective breeding and crossing for successive generations (Guo et al. 2017; Bei et al. 2019; Ghaleb et al. 2020). The new “autotetraploid rice” displayed high fertility (>80%) and high heterosis while crossed with other autotetraploid rice lines having low fertility (Guo et al. 2017; Chen et al. 2018; Bei et al. 2019). Moreover, F<sub>2</sub> and F<sub>3</sub> populations also displayed high fertility and stable morphological traits like neo-*Arabidopsis* (Yu et al. 2009; Guo et al. 2017; Bei et al. 2019). Notably, the new “autotetraploid rice” wasn't an allotetraploid rice;

however, its chromosome behavior was nearly normal, which contributed to high fertility and harbors specific DNA mutations that were different from autotetraploid rice. Therefore, we defined new “autotetraploid rice” as neo-tetraploid rice (Guo et al. 2017). Neo-tetraploid rice is a created new tetraploid rice lines with normal fertility and is a key step to overcome the sterility of  $F_1$  hybrids in tetraploid rice. Our group has reported three neo-tetraploid rice materials that could overcome the sterility of autotetraploid rice and produce high heterosis (Guo et al. 2017; Chen et al. 2018; Bei et al. 2019). However, little is known regarding the complex regulatory mechanisms of heterosis and fertility in neo-tetraploid rice.

High-throughput technologies, such as whole-genome re-sequencing and transcriptome analysis, can provide useful insight for detecting genetic variation in rice. Using whole-genome re-sequencing, a high number of sequence polymorphisms, including single-nucleotide polymorphisms (SNPs) and insertions/deletions (Indels), can be detected (Huang et al. 2013; Varshney et al. 2009). SNPs and Indels within a genome affect gene expression and can alter gene function. Therefore, detecting genomic polymorphisms relevant to functional changes is important for elucidating phenotypic differences. Extensive genome-wide studies using high-throughput technologies to identify SNPs and Indels have identified phenotypic variations and variation in gene expression and function in rice. In autotetraploid rice, very few studies have focused on the relationship between the genetic variations within the genome and pollen fertility (Guo et al. 2017; Li et al. 2018; Bei et al. 2019).

Neo-tetraploid rice is thought to be one effective way to overcome the low fertility of autotetraploid rice; therefore, understanding the mechanism of high fertility in neo-tetraploid rice is important. In this study, we developed a new neo-tetraploid rice, named Huaduo1 (H1), which was registered for Protection for New Varieties of Plants in China in 2016. We used cytological analysis, whole-genome re-sequencing and RNA-sequencing analysis to analyse the mechanism of high pollen fertility in neo-tetraploid rice with respect to that of its two parents. Cytological analysis was used to compare the phenotypic differences between the neo-tetraploid rice and its parents. Whole-genome re-sequencing and transcriptional analysis were used to discover that a large number of differentially expressed genes resulted in high fertility in neo-tetraploid rice. Further, to analyse the relationship between the up-regulated pollen fertility genes and high fertility in neo-tetraploid rice, we selected the representative gene to verify our hypothesis. The results of this study may help to understand the molecular mechanism of high fertility in neo-tetraploid rice.

## Results

### Breeding procedure of neo-tetraploid rice Huaduo1

A hybrid of two autotetraploid rice plants, Jackson-4x (T45-4x) and 96025-4x (T44-4x), was generated in 2004, and the  $F_1$  hybrid plants were harvested and continuously self-crossed until  $F_5$  in 2007 (Fig. 1). One line with more than 80% seed setting was found in that year, and a neo-tetraploid rice line, Huaduo1 (H1), was developed in 2009 and registered for PVP (Protection for New Varieties of Plants) in China in 2016 (Fig. 1). H1 displayed significant differences in agronomic traits compared with its parent, which included high pollen fertility (95.62%) and seed setting (80%) (Fig. 1; Tables 1 and 2). Moreover, H1 also showed significant heterosis in yield-related traits, including number of filled seeds, 1000 seed weight and seed setting (Additional file 1: Table S1).

To evaluate the ability to overcome the sterility of hybrids in H1, we developed hybrids with gene interactions in pollen sterility loci, *Sa*, *Sb* and *Sc* using H1 crossed with Taichung 65-4x and its pollen sterility isogenic lines (Additional file 2: Table S2). All of the hybrids had a high seed setting (>70%) with gene interactions in pollen sterility loci, *Sa*, *Sb* and *Sc* (Additional file 2: Table S2), suggesting that H1 may have neutral pollen fertility genes that could

overcome the sterility of hybrids. All of these results indicate that H1 exhibited significant phenotypic variation compared its two parents and have the potential to overcome the sterility of autotetraploid rice hybrids.

### **Meiosis in neo-tetraploid rice compared with its two parents**

As for the important role of meiosis in autotetraploid rice, we focused on the chromosomal behaviour of PMCs in H1 and its parents. Similar meiotic processes and stage divisions were found in neo-tetraploid rice and their parents, which is consistent with our previous study (Fig. 2). A total of six key meiosis stages, including Metaphase I, Anaphase I, Telophase I, Metaphase II, Anaphase II and Telophase II, were observed, and the percentage of abnormalities is summarized (Fig. 3; Additional file 3: Table S3; Additional file 4: Table S4). H1 showed a lower percentage of abnormal PMCs than those of its two parents (Fig. 3). In this study, the percentages of abnormal cells in neo-tetraploid rice were 19.48, 1.61, 1.80, 13.64, 34.29 and 1.94% in Metaphase I, Anaphase I, Telophase I, Metaphase II, Anaphase II and Telophase II, respectively (Additional file 3: Table S3; Additional file 4: Table S4). In contrast, the autotetraploid rice parents of T45-4x and T44-4x showed many more abnormalities than the neo-tetraploid rice (Fig. 3). For example, the percentages of abnormal cells in T44-4x were 52.14, 15.05, 3.96, 23.10, 52.14 and 12.12% in Metaphase I, Anaphase I, Telophase I, Metaphase II, Anaphase II and Telophase II, respectively (Additional file 3: Table S3; Additional file 4: Table S4).

Additionally, chromosome configurations between H1 and its two parents were significantly different (Fig. 2). Higher percentages of quadrivalent and bivalent configurations were frequently observed in H1 relative to those in its two parents (Additional file 5: Table S5). However, chromosome configurations in T45-4x and T44-4x exhibited a much more complicated pairing style, such as univalent, trivalent and other types of multivalent (Additional file 5: Table S5). All of these results indicate that H1 had a higher percentage of normal cells and more stable chromosome configurations than those of their parents during meiosis.

### **Genome-wide alterations reveal the genetic variation of pollen fertility genes in neo-tetraploid rice and its parents**

To reveal the cause of the higher pollen fertility in neo-tetraploid rice, we used re-sequencing analysis to detect whole genome variation between the neo-tetraploid rice and its two parents. A total of 68.20 GB of high-quality clean reads were obtained from H1 and its parents using the Illumina sequencing platform. Large numbers of high-quality reads varying from 214 to 251 million were obtained in these tetraploid lines (Additional file 6: Table S6). From this study, nearly 87.87% of the reads mapped to the *japonica* rice genome (Nipponbare), which covered ~85% of the total genome for each cultivar (Additional file 6: Table S6).

Two types of genetic variation, including the non-synonymous SNP mutations and Indels of the CDS region, were conducted in this study for their important role in influencing the gene expression of relevant proteins. We focused on genetic variations of H1 and divided it into three groups based on their origin which deriving from two parents or non-parental variations. In this study, Group I is termed to the genetic variation in neo-tetraploid rice derived from the T45-4x, and detected by the comparison between (H1 vs T44-4x) and (T45-4x vs T44-4x). Group II is termed to the genetic variation only from the T44-4x, and detected by the comparison between (H1 vs T45-4x) and (T45-4x vs T44-4x). Group III is termed to the non-parental variations of neo-tetraploid rice (Additional file 7: Table S7). We compared the genome-wide alterations of SNP mutations and Indels in neo-tetraploid rice with its two parents across the 12 rice chromosomes (Additional file 8: Figure S1). Results showed that a total of 2631 SNPs and 566 Indels involved in 1509 genes were predicted to have differences between neo-tetraploid rice and its two parents. These results indicated that genetic variations from T45-4x, T44-4x and non-parental variations exhibited the similar tendency, and SNPs and Indels variations were primary occur on the Chr1, Chr5, Chr6 and Chr12 (Fig. 4).

We further combined these genetic variations with pollen fertility-related genes, such as pollen fertility candidate QTLs and pollen fertility-related genes. From this study, a total of 29 fertility candidate QTLs and 113 pollen fertility-related genes were found to exhibit genetic variation in neo-tetraploid rice compared with its parents (Additional file 9: Table S8). To validate this genetic variation data in neo-tetraploid rice, we selected nine pollen fertility genes and used Sanger sequencing to detect the genetic variation between the neo-tetraploid rice and its two parents. From this study, genetic variation between the neo-tetraploid rice and its two parents was also consistent with the re-sequencing analysis (Additional file 10: Table S9).

### **Transcriptome analysis reveals that significant variation exists in neo-tetraploid rice compared with its two parents in meiosis**

We further combined the transcriptome profiling with re-sequencing data to detect possible variations in gene expression that could result in the high pollen fertility seen in meiosis. Three comparison groups were used to identify the possible DEGs between the neo-tetraploid rice and its parents. Group I is termed “genome variation of re-sequencing data between the neo-tetraploid rice and its parents”, referring to possible genetic variation for the non-synonymous SNP mutations and Indels of the CDS region, which might influence the gene expression of relevant proteins in neo-tetraploid rice. Group II is termed “differential gene expression of transcriptome data between the neo-tetraploid rice and its parents”, referring to genes that were differentially expressed in the neo-tetraploid rice and its parents in meiosis. Group III is related to the genes found to demonstrate genetic variation in the genome and that are also differentially expressed at the transcriptional level (Fig. 5).

Using these comparison groups, we have identified a total of 1479 genes that are differentially expressed (2-fold at P value<0.05) between the neo-tetraploid rice and its parents in meiosis (Fig. 5a; Additional file 11: Table S10). Among these DEGs, 785 genes are upregulated in the neo-tetraploid rice relative to its parents, and 694 genes are downregulated (Fig. 5b). We then categorized both the up- and downregulated genes using Cluster 3.0 software and obtained an overview of the transcriptome relationships (Fig. 5c).

Gene ontology (GO) analysis was conducted to annotate the up- and downregulated genes that were differentially expressed between the neo-tetraploid rice and its parents (Additional file 12: Figure S2). The GO enrichment classification suggested that the genes from the cellular component, molecular function and biological process categories showed significant variation. In the biological process category, eight prominent functional gene classes, including cell division, response to auxin, reproductive process, reproduction, pollination, pollen exine formation, pollen-pistil interaction and recognition of pollen, were over-represented in the upregulated gene classes. Additionally, cell cycle, purine nucleobase biosynthetic process and regulation of protein dephosphorylation were over-represented in the downregulated gene classes (Additional file 12: Figure S2a). In the cellular component category, one prominent functional gene class, the vacuolar membrane, was over-represented in the upregulated gene classes, and three prominent functional gene classes, apoplast, chloroplast thylakoid membrane and microtubule-associated complex, were over-represented in the downregulated gene classes (Additional file 12: Figure S2b). In the molecular function category, four prominent functional gene classes, transcription regulator activity, DNA binding transcription regulator activity, obsolete transcription regulator activity, and cation binding, were over-represented in the upregulated gene classes, and seven prominent functional gene classes, including protein kinase binding, peptidase inhibitor activity, xyloglucan xyloglucosyl transferase activity, hydrolase activity, peptide receptor activity, transmembrane signalling receptor activity and motor activity, were over-represented in the downregulated genes (Additional file 12: Figure S2c). All of these results indicate that the upregulated genes were mainly involved in response processing and

transcription regulator activity-related genes. Alternatively, the downregulated genes were mainly involved in the external encapsulating structure and cell wall-related genes.

### **Pollen fertility-related genes are primarily upregulated in neo-tetraploid rice compared with its two parents**

RNA-sequencing and re-sequencing analysis were used to reveal the cause of the higher pollen fertility in the neo-tetraploid rice; thus, we focused on the pollen fertility-related genes that were differentially expressed in neo-tetraploid rice compared with its two parents. We compared our DEGs detected from neo-tetraploid rice and its two parents with the large number of rice pollen fertility-related genes (Fujita et al. 2010; Deveshwar et al. 2011; Yant et al. 2013; Wright et al. 2015; Wu et al. 2014).

Of these DEGs, 433 genes were annotated as pollen fertility genes when combined with other analysis results (Fig. 6). Notably, 240 of the pollen fertility genes were shown to be upregulated in neo-tetraploid rice compared with its two parents (Fig. 7; Additional file 13: Table S11). Predicted protein-protein interaction analysis was used to further evaluate the relationship of these pollen fertility genes. From this study, a strong interaction network was detected in the neo-tetraploid rice (Additional file 14: Figure S3). Moreover, several pollen fertility genes, such as *LOC\_Os01g16810*, *LOC\_Os02g12290*, *LOC\_Os04g53760*, *LOC\_Os04g37960*, *LOC\_Os09g27620*, *LOC\_Os03g12414*, *LOC\_Os06g49840*, *LOC\_Os07g39220*, *LOC\_Os07g30240* and *LOC\_Os10g35180*, were detected and upregulated in the neo-tetraploid rice. It is noted that three genes, *CSA* (*LOC\_Os01g16810*), *TMS5* (*LOC\_Os02g12290*) and *TMS9-1* (*LOC\_Os09g27620*), are pollen fertility-related genes that are also responsive to the environment. *CSA* (*LOC\_Os01g16810*) is a *MYB* family transcription factor encoding a MYB protein domain that plays an important role in the pollen development process under the conditions of a short day. *TMS5* (*LOC\_Os02g12290*) is a nuclear ribonuclease Z that processes the mRNAs of three *ubiquitin fusion ribosomal protein L40* (*Ubl40*) genes into multiple fragments, which could result in pollen sterility under high temperature. *TMS9-1* is a transcript factor containing a PHD-finger domain that controls pollen sterility under high temperature. To verify the expression profiles of pollen fertility genes in neo-tetraploid rice and its two parents, nine representative pollen fertility-related genes were selected and submitted to quantitative real-time reverse transcription PCR (qRT-PCR) analysis. From this study, nine genes, including *LOC\_Os01g16810*, *LOC\_Os04g53760*, *LOC\_Os04g37960*, *LOC\_Os09g27620*, *LOC\_Os03g12414*, *LOC\_Os06g49840*, *LOC\_Os07g39220*, *LOC\_Os07g30240* and *LOC\_Os10g35180*, were consistent with the transcriptome analysis. These results showed that expression levels of the nine genes were consistent with the transcriptome analysis, indicating the reliability and accuracy of RNA-sequencing results (Additional file 15: Figure S4).

### **Knock-out of environmentally sensitive genic male sterility genes causes pollen abortion in neo-tetraploid rice**

Neo-tetraploid rice is a key step to overcome the sterility of F<sub>1</sub> hybrids in autotetraploid rice (Guo et al. 2017; Bei et al. 2019; Mo et al. 2020). To verify the upregulation of pollen fertility genes play the important role in neo-tetraploid rice, we used CRISPR/cas9 technology to conduct the study. In this study, we selected two environmentally sensitive genic male sterility candidate genes, named *TMS5* and *TMS9-1*, as these genes not only found to be up-regulation in neo-tetraploid rice but also play the important role hybrid cross (Additional file 16: Figure S5) and it will provide a possibility to utilize the greater hybrid vigor in polyploidy rice. In the present work, we obtained at least 20 independently regenerated transgenic lines of *TMS5* and *TMS9-1* after the transformation, respectively (Additional file 17: Figure S6; Additional file 18: Figure S7). The mutant lines were grown in the field, and the T<sub>2</sub> mutants were sequenced.

In this study, we selected one homozygous mutant, named *nt-tms5-1* and its sequencing results indicate that it carried the sequence deletion that was predicted to lead to an amino acid change (Additional file 17: Figure S6). We grew the plants in August at an LD and under high temperature conditions to verify their pollen fertility. Both *nt-tms5-1* and its wild type showed marked differences in anther, panicle and pollens (Fig. 8). Pollen fertility in *TMS5* knockout lines (*nt-tms5-1*) showed higher pollen sterility, and its value was notably reduced compared with that of its wild type (Fig. 8). The statistical analysis results demonstrated that the pollen fertility value of *nt-tms5-1* was much lower than that of its wild type. Moreover, Whole-mount eosin B-staining confocal laser scanning microscopy (WE-CLSM) analysis of anther in *nt-tms5-1* verified that the number of normal pollen was obviously decreased compared with that of the wild type (Fig. 8).

Anther development was investigated further for *nt-tms5-1* and its wild type H1. The results indicate that the anther development process of *nt-tms5-1* was similar to its wild type (Fig. 8). Anther development in *nt-tms5-1* was primarily divided into eight differential stages: pollen mother cell formation, meiosis, the early, middle, and late microspore stages, and the early bicellular, late bicellular and mature pollen stages. In the wild type, a four-layer anther wall (from the outside to the inside: epidermis, endothecium layer, middle layer, and tapetum) was generated at the pollen mother cell formation stage. No obvious defects were found between the WT and *nt-tms5-1* anthers in the formation of PMCs. During pollen mother cell (PMC) meiosis, the PMCs underwent meiosis, and normally formed dyads with cell plates in the WT and *nt-tms5-1* were clearly observed (Fig. 8). Subsequently, dyads also formed in *nt-tms5-1*, and no abnormalities were found in this stage (Fig. 8). Thereafter, the microspores of WT underwent vacuolation and mitosis to form mature pollen with spherical or elliptical shapes (Fig. 8). In contrast, the microspores of *nt-tms5-1* degraded further after the late microspore stage and completely disappeared at the mature pollen stage, which resulted in an empty anther locule (Fig. 8). All of these results suggest that the lack of *TMS5* also causes the defects in the microspores as well abnormal pollen in the neo-tetraploid rice during the pollen development.

To further investigate how *TMS5* works in regulating the pollen development process of neo-tetraploid rice, 10 genes were selected to analyse their gene expression levels in *nt-tms5-1* and its wild type (Fig. 9a). We conducted the qRT-PCR analysis to detect the nine transcripts of these genes in both *nt-tms5-1* and its wild type (Fig. 9b). In this study, the expression levels of the nine genes were shown to be differentially expressed between *nt-tms5-1* and its wild type. For example, four genes, *LOC\_Os04g50600*, *LOC\_Os04g32930*, *LOC\_Os03g40110*, and *LOC\_Os04g59600*, were downregulated in the meiosis stage in *nt-tms5-1* compared to their levels in the wild type. Five genes, including *LOC\_Os06g43690*, *LOC\_Os03g13160*, *LOC\_Os03g05720*, *LOC\_Os09g30486* and *LOC\_Os04g46310*, were upregulated in meiosis in *nt-tms5-1* compared to those of the wild type. These results verify that *TMS5* have strong interaction with other genes and knockout of *TMS5* could also down regulation of other genes in neo-tetraploid rice.

## Discussion

### Significant phenotypic variation exists in the neo-tetraploid rice compared with its two parents

Autotetraploid rice is a new germplasm resource derived from diploid rice by chromosome doubling. Abundant advantages, such as a stronger stem, wider leaf and bigger grains, exist in the autotetraploid rice compared to those of its diploid counterparts (Tu et al. 2007; Wu et al. 2013; Wu et al. 2014). Agronomic traits in autotetraploid rice demonstrate significant potential to improve the rice biomass yield (Tu et al. 2007; Shahid et al. 2011; Wu et al. 2013). However, lower fertility of autotetraploid rice is still an important issue for utilizing its potential vigour. It took us more than twenty years to generate the neo-tetraploid rice, and we found that it was one type of the stable

autotetraploid rice lines derived from the progeny of autotetraploid rice (Guo et al. 2017; Bei et al. 2019). In our previous analysis, we proposed that neo-tetraploid rice have higher fertility and hybrid vigour, which could overcome the low fertility of autotetraploid rice (Guo et al. 2017; Bei et al. 2019). Therefore, it is of greater value to evaluate the phenotypic variation of the neo-tetraploid rice compared with its two parents.

In the present work, one of newly developed neo-tetraploid rice lines, named Huaduo 1 (H1), which has been registered for PVP in China, was used. We analysed the phenotypic variation of H1 and detected that three of the seven primary agronomic traits, including the plant height, seed set ratio and 1000-grain weight, varied significantly compared to those of the two parents. Notably, the seed set ratio in the neo-tetraploid rice can reach >80%, which is much higher than that of its two parents. These obvious phenotypic variations were similar to the other type of neo-tetraploid rice (Bei et al. 2019) and show great potential for the utilization of H1. Additionally, we also evaluated the heterosis and gene interaction effect using Taichung 65-4x and its pollen sterility near-isogenic lines and found that the seed set ratio of the hybrids can reach to more than 80%. These results show the significant potential that H1 may have given the neutral genes for pollen fertility that could overcome the sterility of hybrids. The neutral genes could overcome the hybrid's sterility caused by the multi-pollen sterility loci interactions in autotetraploid rice hybrids (Wu et al. 2017; Chen et al. 2019).

Pollen fertility was thought to be the important factor for determining production in autotetraploid rice. Abnormal meiotic chromosome behaviour, microtubules or interactions of pollen sterility-related genes were the primary reasons leading to pollen abortion in autotetraploid rice (He et al. 2011; Wu et al. 2014; Li et al. 2018). Therefore, we observed the chromosome behaviour of PMCs to detect the genetic variations between neo-tetraploid rice and its parents. The results indicate that the percentage of abnormalities in PMCs was higher in T44-4x and T45-4x compared with that in the neo-tetraploid rice. For example, the percentages of abnormal cells in T44-4x is 52.14, 15.05, 3.96, 23.10, 52.14 and 12.12% in Metaphase I, Anaphase I, Telophase I, Metaphase II, Anaphase II and Telophase II, respectively. In contrast, the percentages of abnormal cells in H1 were much lower than those of its two parents. Additionally, one interesting phenomenon was found in the configuration of the neo-tetraploid rice at the diakinesis stage. The configuration in neo-tetraploid rice was primarily bivalent and quadrivalent and showed significant differences from its two parents. All of these results indicate that neo-tetraploid rice shows obvious variation and a higher percentage of fertility than its two parents.

### **Upregulation of pollen fertility genes plays is critical for high pollen fertility in neo-tetraploid rice**

Neo-tetraploid rice exhibit higher fertility and greater heterosis than those of the autotetraploid rice lines (Guo et al. 2017; Bei et al. 2019); however, there are still limitations to understanding the high fertility mechanism of neo-tetraploid rice. To understand the regulatory mechanism of high fertility in neo-tetraploid rice, we combined the re-sequencing and RNA-sequencing analysis to analyse the genomic and transcriptional variations between neo-tetraploid rice and its two parents. Using the transcriptome analysis profile, we identified a total of 1479 DEGs that showed at least a 2-fold change in neo-tetraploid rice compared with its two parents. Gene ontology (GO) analysis was conducted to annotate the up- and downregulated genes that were differentially expressed in neo-tetraploid rice and its parents in this study. The GO enrichment classification indicated that differentially expressed genes in the cellular component, molecular function and biological process categories showed significant variations. We found that up-regulated differentially expressed genes were mainly involved in the response process, pollen development and transcription regulator activity.

Meiosis is an important process, where errors can result in low fertility in autotetraploid rice. Meiosis-related and pollen fertility genes were frequently detected to be downregulated, and this was thought to be the primary cause of

the lower pollen fertility in autotetraploid rice (Wu et al. 2014; Wu et al. 2017; Wu et al. 2019). Compared with autotetraploid rice, pollen fertility and seed set of neo-tetraploid rice were much higher and exhibited normality (Guo et al. 2017; Bei et al. 2019). We speculated that some important pollen fertility-related genes may be expressed normally again or upregulated in neo-tetraploid rice when compared with autotetraploid rice. Therefore, we focused on differentially expressed genes that were upregulated in neo-tetraploid rice compared with autotetraploid rice.

In the present work, we combined our results with the pollen fertility genes detected by previous analyses (Fujita et al. 2010; Deveshwar et al. 2011; Hollister et al. 2012; Yant et al. 2013; Wu et al. 2014; Wright et al. 2015; Wu et al. 2015). A total of 240 pollen fertility genes were detected and found to be upregulated in neo-tetraploid rice compared with its two parents. Notably, 93 pollen fertility genes were detected in Wu et al. (2014), and 22 pollen fertility genes showed a similar tendency as those by Guo et al. (2017). These results verify our transcriptome analysis data and indicate that pollen fertility genes play an important role in neo-tetraploid rice. Moreover, pollen fertility genes in the neo-tetraploid rice of H1 were found to involve a strong network based on the predicated protein-protein interaction analysis.

Among these pollen fertility genes, ten pollen fertility related genes, including *LOC\_Os01g16810*, *LOC\_Os02g12290*, *LOC\_Os04g53760*, *LOC\_Os04g37960*, *LOC\_Os09g27620*, *LOC\_Os03g12414*, *LOC\_Os06g49840*, *LOC\_Os07g39220*, *LOC\_Os07g30240* and *LOC\_Os10g35180*, encoded the meiosis-related proteins that are mainly involved in the rice pollen development process. Notably, several genes have been functionally analysed or predicted that show a relationship in pollen development. Notably, three genes, *CSA* (*LOC\_Os01g16810*), *TMS5* (*LOC\_Os02g12290*) and *TMS9-1* (*LOC\_Os09g27620*), are pollen fertility-related genes that are responsive to temperature. *CSA* (*LOC\_Os01g16810*) is a *MYB* family transcription factor that encodes a *MYB* protein domain, which plays an important role in the pollen development process. *TMS5* (*LOC\_Os02g12290*) is a nuclear ribonuclease Z that processes the mRNAs of three ubiquitin fusion ribosomal protein L40 (Ubl40) genes into multiple fragments, which could result in pollen sterility under low temperature. *TMS9-1* (*LOC\_Os09g27620*) is a male sterility gene that responsive to the temperature (Qi et al. 2014). All of these results indicate that up-regulation of pollen fertility genes plays an important role in the high fertility of neo-tetraploid rice.

### **Environmentally sensitive genic male sterility (EGMS) likely regulates pollen fertility in neo-tetraploid rice**

High pollen fertility is a primary characteristic of neo-tetraploid rice; therefore, it is of great value to understand the mechanism of its higher pollen fertility. With the advantage of effective tools such as CRISPR/Cas9 technology, we are given the opportunity to knock out important fertility-related genes. To date, there is no information regarding the effect of knocking out important fertility genes in neo-tetraploid rice. In the present work, we detected three environmentally sensitive genic male sterile genes, *CSA* (*LOC\_Os01g16810*), *TMS5* (*LOC\_Os02g12290*) and *TMS9-1* (*LOC\_Os09g27620*), that were upregulated in neo-tetraploid rice compared with its two parents. We proposed that pollen fertility-related genes may be upregulated or normally expressed in neo-tetraploid rice compared with those of the autotetraploid rice. Therefore, we selected two important genes, *TMS5*, and *TMS9-1* to verify our speculation in neo-tetraploid rice. *TMS5* is an important photoperiod- and thermo-sensitive gene that can lead to the TGMS trait through a loss of RNase Z<sup>S1</sup> function (Zhou et al. 2014). *TMS9-1* is a male sterility gene that responsive to the temperature (Qi et al. 2014).

From this study, we obtained a homozygous mutant of *TMS5* and *TMS9-1*. Our results indicated that both *nt-tms5-1* and *nt-tms9-1* exhibited marked differences in anther, panicle and pollen compared with its wild type. Moreover, we focused on pollen fertility and pollen development experienced differential variation during the pollen development stage in *nt-tms5-1*. *TMS5* also has similar results and exhibited pollen sterility under the conditions of a long day. As

*TMS5* is an important photoperiod- and thermo-sensitive gene, this method also provides the possibility for boosting the development of excellent pollen sterility lines or revealing the mechanism of high pollen fertility in neo-tetraploid rice. In the future, better yield and quality can also be developed by editing important pollen fertility genes for fertility and disease at the same time in neo-tetraploid rice in the appropriate genetic background.

## Conclusions

In the present study, we found that upregulation of pollen fertility genes plays an important role in neo-tetraploid rice. Our results provide strong evidence that the upregulation of pollen fertility genes results in high fertility of neo-tetraploid rice using cytological and transcriptome analysis. Differentially expressed genes, including 240 upregulated pollen fertility genes, can be used as candidate genes to reveal the mechanism of high pollen fertility in neo-tetraploid rice in the future.

## Methods

### Plant material

Three materials, including the H1 and its two parent (T44-4x and T45-4x), were used in this study. T44-4x and T45-4x are autotetraploid rice, and produced by artificial polyploidization with colchicine. H1 was the high pollen fertility material derived from the hybrid crosses from T44-4x and T45-4x and then self-crossed for more than 15 generations. All of these materials were planted under the natural conditions at the experimental farm of South China Agricultural University (SCAU) and standard practices followed the recommendations for the area.

### Analysis of agronomic traits and heterosis

To detect the genetic variation of neo-tetraploid rice and its two parent, total eight agronomic traits were selected to detect the phenotypic variation, i.e. plant height (PH, cm), panicle length (PL, cm), effective panicles number (EPN), panicle length (PL, cm), total number of grains per plant (TGP), 1000-grain weight (GWT, g) and seed set ratio ( $SS = (\text{number of filled grains}/\text{total number of grains}) \times 100$ ). These traits were detected according to our previous study (Wu et al. 2013).

Heterosis analysis was conducted to evaluate the heterosis level of H1, total 13 differential parents crossed with the H1 and  $F_1$  hybrid. High-parent heterosis (HPH) and mid-parent heterosis (MPH) were estimated by the following formula:  $HPH = (F_1 - HP)/HP \times 100\%$ , and  $MPH = (F_1 - MP)/MP \times 100\%$ , where  $F_1$  is the performance of first filial generation (hybrid), HP is the performance of the best parent, and MP is the average performance of two parents.

### Pollen fertility, chromosome behavior observation

Pollen fertility of H1 and its two parents was observed according to our previous study with minor modifications (Shahid et al. 2013). More than 1000 pollen grains were calculated for pollen fertility under a microscope (Motic BA200).

The meiosis chromosome behaviour experiment was performed according to Wu et al. (2014). To observe the chromosome behaviour in the meiosis process, samples were collected from the shoots of rice plants with -2 to 2 cm between their flag leaf cushion and the second-to-last leaf cushion. Then, the samples were fixed in Carnoy's solution (ethanol:acetic acid, 3:1 v/v) for at least 24 h and washed using 95% and 80% ethanol for ~30 min each.

Finally, they were washed and kept in 70% ethanol at 4°C until observation. The meiosis chromosome behaviour and meiosis stage divisions were observed according procedures described by He et al. (2011a) and Wu et al. (2014).

### **DNA library construction, massive re-sequencing, and validation analysis**

Genomic DNA of three materials, H1, T44-4x and T45-4x, were extracted from fresh leaves according to the procedure described by Chen et al. (2018). Sequencing libraries were constructed from genomic DNA of neo-tetraploid rice and its two parents and sequenced on an Illumina HiSeq™2500 according to the manufacturer's instructions. Whole genome re-sequencing analysis was performed by Biomarker Technologies (Beijing, China) with an average coverage of approximately 45× in each material. The sequencing reads were aligned to the *japonica* Nipponbare reference genome using BWA software. Identification of polymorphic sites, including SNPs and indel analyses, between neo-tetraploid rice and its two parents was performed with GATK software tools. SNPs and indel annotations were performed using SnpEff software.

Polymerase chain reaction (PCR) was conducted to verify the re-sequencing results using the genomic DNA of neo-tetraploid rice and its two parents as templates. Important polymorphic DNA of pollen fertility-related genes were selected in this study. Primers were designed using Primer Premier 5.0 software, and the product length ranged from 400 to 800 bp (Additional file 19: Table S12a). The whole PCR program was 94 °C for 5 min followed by 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were sequenced, and sequence variations were detected using BioEdit software.

### **Sample preparation, RNA extraction, RNA-sequencing, and qRT-PCR verification**

Anthers from pre-meiotic to prophase I of meiosis stage were confirmed by fluorescence microscope. All samples (H1, T44-4x and T45-4x) were collected in three biological replicates and stored at -80°C until RNA extraction. Total RNA of each sample was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The quantity and purity of total RNA were analysis by Bioanalyzer 2100 and RNA 6000 Nano Lab Chip Kit (Agilent, CA, USA) with RIN number > 7.0. RNA-sequencing library preparation was carried out according to the manufacturer's protocol and performed on the Illumina HiSeq™2500 by Biomarker Technologies (Beijing, China). Genes with  $FC \geq 2$  (fold change) and  $FDR \leq 0.01$  were chosen for the t-test, and genes with  $P \text{ values} < 0.05$  were chosen for further analysis. After selected the differentially expressed genes, cluster analysis and GO enrichment analysis were conducted using the Cluster 3.0 software and agriGO (Du et al. 2010). Venny software was used to identify the overlapped differentially expressed genes in different samples (<http://bioinfogp.cnb.csic.es/tools/venny/>).

Real-time qRT-PCR analysis was conducted to examine the expression patterns of neo-tetraploid rice and its two parents. Total twelve candidate genes were selected and used to validate the transcriptome data using the same RNA samples of RNA-sequencing (Additional file 19: Table S12b). Reverse transcription reaction was done using the Roche Transcriptor First Strand cDNA Synthesis kit. The qRT-PCRs experiment was performed on the Lightcycler480 system (Roche) using the Advanced SYBR Green Supermix Kit (Bio-RAD). The qRT-PCR cycles were using the following reaction conditions: 95°C for 30s, 40 cycles of 95°C denaturation for 5s and 58°C annealing and extension for 20s. All qRT-PCR reactions were performed in triplicate, and the results were calculated using the  $2^{-\Delta\Delta Ct}$  method. Rice ubiquitin gene used as an internal control to normalize the expression levels. Each PCR reaction repeated three times.

### **Generation and mutation detection of mutant plants in neo-tetraploid rice**

We used the CRISPR-Cas9 binary vector pC1300-cas9 to knock out *TMS5* and *TMS9-1* obtained the *nt-tms5-1* and *nt-tms9-1* mutant. The knockout lines derived from H1 using the CRISPR/cas9 technique and construct was introduced to EHA105 and then transformed into H1 to generate the *nt-tms5-1* and *nt-tms9-1* mutant line. We extracted the genomic DNA from transformants, and the genomic DNA was sequenced for mutant identification. The PCR products (500-800 bp) were sequenced and identified using the De-generate Sequence Decoding method. Mutations were identified by comparing the amplicon sequences derived from putative transgenic and pC1300-cas9 templates.

### **Semi-thin section, WE-CLSM analysis, and expression analysis of *TMS5* plants in neo-tetraploid rice**

Samples of *nt-tms5-1* mutant and its wild type were fixed in FAA solution for 48 h. After being washed in 50% ethanol several times, the samples were dehydrated in a series of ethanol solutions and then embedded by a Leica 7022 historesin embedding kit (Leica, Nussloch, Germany) according to the manufacturer's instructions. The embedded samples were further sectioned using the Leica RM2235 manual rotary microtome, stained with 1% toluidine blue O and sealed with neutral balsam.

WE-CLSM analysis was used to detect the phenotypic variation of H1 and *nt-tms5-1*. Anthers and mature pollens were stained using a small drop of 10 mg/L eosin B (C20H6N2O9Br2Na2, FW 624.1, a tissue stain for cell granules and nucleoli) solution (dissolved in 4% sucrose) on a glass slide. After 10 min, the glass slide was covered with a slide cover and scanned under a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany). The detailed procedures have been described previously (Zeng et al. 2007).

## **Abbreviations**

PMCs: Pollen mother cells; PVP: Protection for New Varieties of Plants; DEGs: Differentially expressed genes; SNPs: Single-nucleotide polymorphisms; Indels: Insertions/deletions; H1: Huaduo1; GO: Gene ontology; WE-CLSM: Whole-mount eosin B-staining confocal laser scanning microscopy; UbL40: Ubiquitin fusion ribosomal protein L40; EGMS: Environmentally sensitive genic male sterility; PH: Plant height; PL: Panicle length; EPN: Effective panicles number; TGP: Total number of grains per plant; SS: Seed set ratio; HPH: High-parent heterosis; MPH: Mid-parent heterosis; PCR: Polymerase chain reaction.

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

### **Availability of data and materials**

All data supporting the conclusions described here are provided in tables, figures, and additional files.

### **Competing interests**

The authors have declared that no competing interests exist.

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## Author Contributions

XDL conceived and designed the experiments. JWW, YMC and XDL wrote the paper. JWW, YMC, LH, YC, HY, ZJL, LX, HZ and ZXC performed the experiments and analyzed the data. XDL and JWW developed Huaduo1. All authors read and approved the final manuscript.

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## Tables

Table 1 Pollen fertility of neo-tetraploid rice and its parents

Material name	Pollen fertility (%±SE)	Typical aborted pollens (%±SE)	Stained aborted pollens (%±SE)	Small pollens (%±SE)
T45-4x	54.10±3.81	8.18±0.72	37.26±3.92	0.46±0.12
T44-4x	57.66±2.78	15.08±1.56	23.07±1.98	4.19±0.51**
H1	95.62±0.34**	3.16±0.34**	0.60±0.13**	0.62±0.12

Note: \*\* indicated significant difference existed in pollen fertility between neo-tetraploid rice and its parents from zero at  $P < 0.01$ .

Table 2 Genetic variation in agronomic traits of neo-tetraploid rice and their parents

Seasons	Material name	PH (%±SE)	EPN (%±SE)	PL (%±SE)	FG (%±SE)	TGP (%±SE)	SS (%±SE)	GWT (%±SE)
Late season	T45-4x	94.40±1.19**	3.40±0.27**	25.97±0.60	63.27±4.78	120.90±9.17*	53.27±2.34**	30.74±1.39*
	T44-4x	78.10±1.58**	5.65±0.44	23.53±0.33	15.58±1.41**	55.89±1.94*	26.45±1.88**	31.39±0.46
	H1	104.40±0.93	6.60±0.40	25.46±0.52	66.34±4.63	87.02±6.34	76.46±1.43	35.44±0.34
Early season	T45-4x	117.70±0.64**	4.45±0.28	31.74±0.46**	40.26±1.33**	132.38±4.05	30.80±0.97**	30.82±1.04**
	T44-4x	96.05±0.46**	5.85±0.27*	28.81±0.34	30.17±0.65**	77.90±0.55**	39.16±7.16**	33.74±0.49
	H1	124.45±0.84	4.95±0.22	28.09±0.24	92.75±3.57	120.41±4.23	76.55±0.84	33.86±0.29

\*, \*\* Significantly different from zero at  $P < 0.05$  and  $P < 0.01$ , respectively.

PH = plant height, EPN = effective panicles number, PL = panicle length, FG = Filled grains, TGP = total number of grains per plant, GWT = 1000-grain weight and SS = seed set ratio.

## Additional Files

**Additional file 1: Table S1.** Heterosis analysis of hybrids generated by the crossing of H1 and autotetraploid rice lines.

**Additional file 2: Table S2.** Seed set ratio of hybrids with genetic interactions at *Sa*, *Sb* and *Sc* loci.

**Additional file 3: Table S3.** Frequency of abnormal chromosome behaviors in neo-tetraploid rice and its parents during the Meiosis I.

**Additional file 4: Table S4.** Frequency of abnormal chromosome behaviors in neo-tetraploid rice and its parents during the Meiosis II.

**Additional file 5: Table S5.** Meiotic chromosome configurations in neo-tetraploid rice and its parents.

**Additional file 6: Table S6.** Summary of the re-sequencing data in Huaduo1 and its two parents.

**Additional file 7: Table S7.** Summary of InDel and SNP in Huaduo1 compared with its two parents.

**Additional file 8: Figure S1.** Chromosome-wide counts distribution of SNP and Indels per 100kb between H1 and its two parents.

**Additional file 9: Table S8.** Summary of the pollen fertility related QTLs and genes in in neo-tetraploid rice compared with its two parents.

**Additional file 10: Table S9.** Validation of re-sequencing variations by Sanger sequencing.

**Additional file 11: Table S10.** Differentially expressed genes in neo-tetraploid rice compared with its parents in meiosis stage.

**Additional file 12: Figure S2.** GO analysis of differentially expressed genes in neo-tetraploid rice comparative its two parents.

**Additional file 13: Table S11.** Functional meiosis-related genes in neo-tetraploid rice compared with its two parents.

**Additional file 14: Figure S3.** Predicted protein-protein interaction network of DEGs specifically expressed in neo-tetraploid rice compared with its parents.

**Additional file 15: Figure S4.** Comparison of the  $\log_2$  (FC) of 12 selected genes using the qRT-PCR analysis.

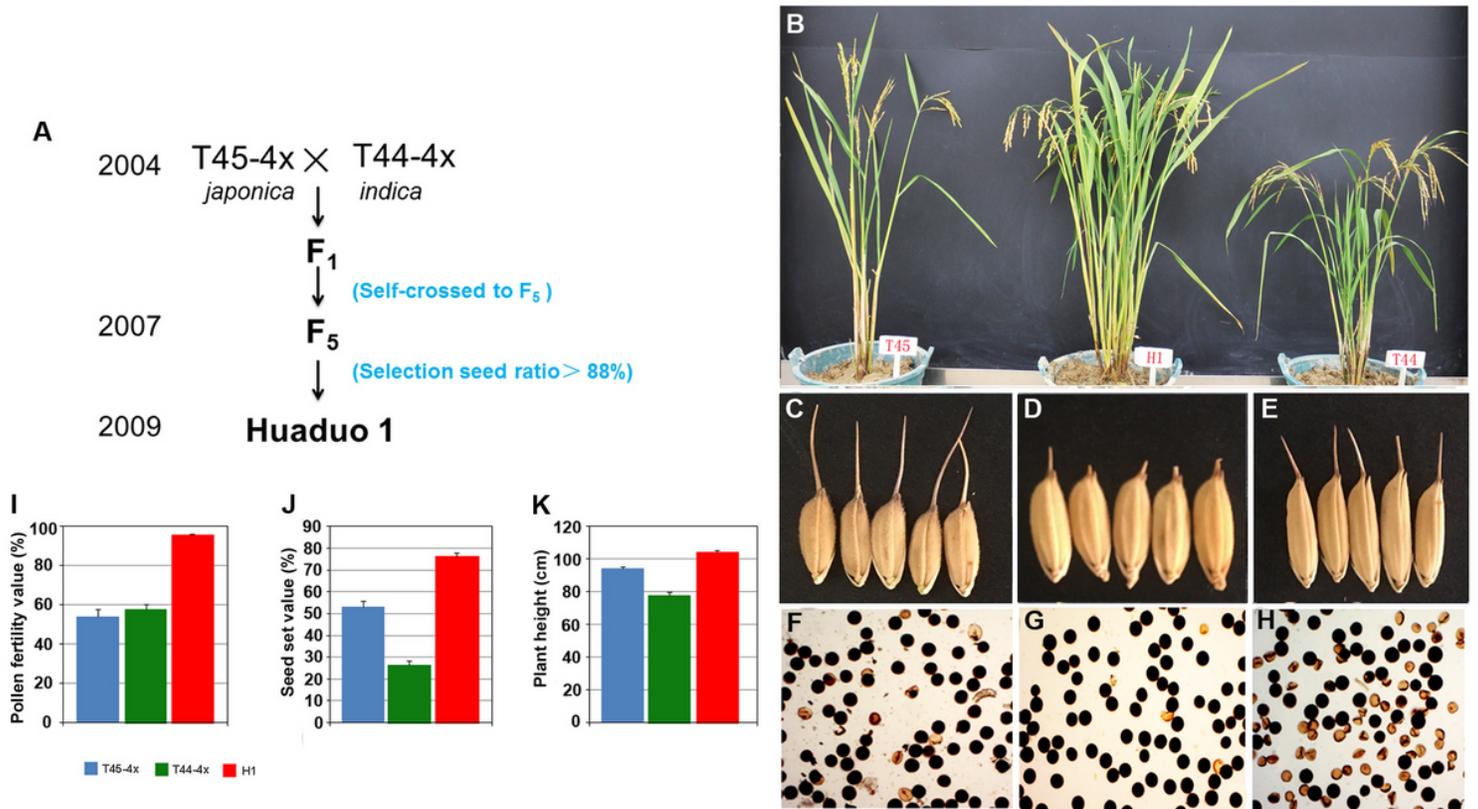
**Additional file 16: Figure S5.** Quantitative real-time PCR confirmation of candidate genes in neo-tetraploid rice and its two parents.

**Additional file 17: Figure S6.** Mutations of *TMS5* target sites and its expression level in Huaduo1 and *nt-tms5*.

**Additional file 18: Figure S7.** Mutations of *TMS9-1* target sites and its phenotype in Huaduo1 and *nt-tms9-1*.

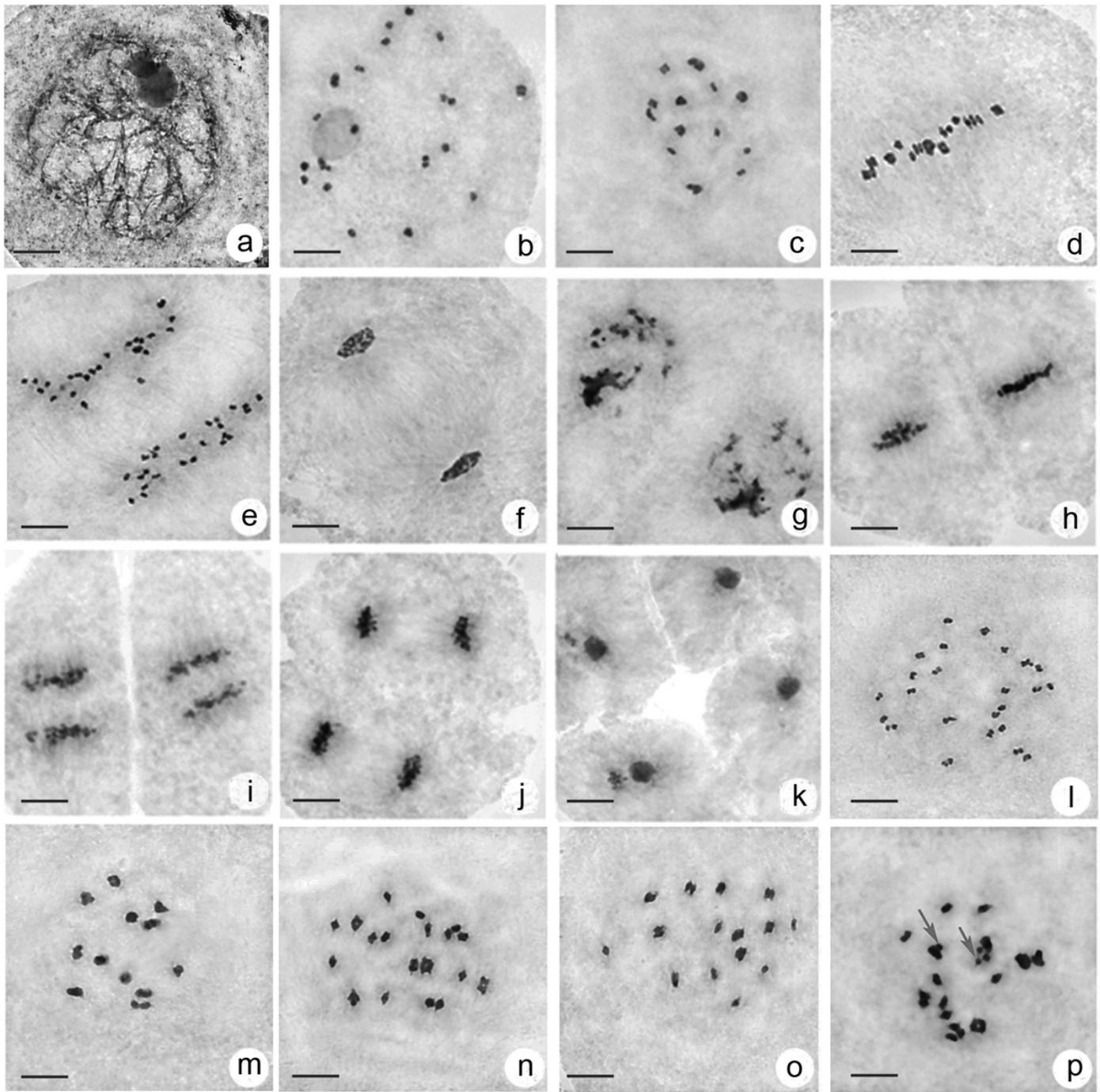
**Additional file 19: Table S12.** List of primers used in this study.

## Figures



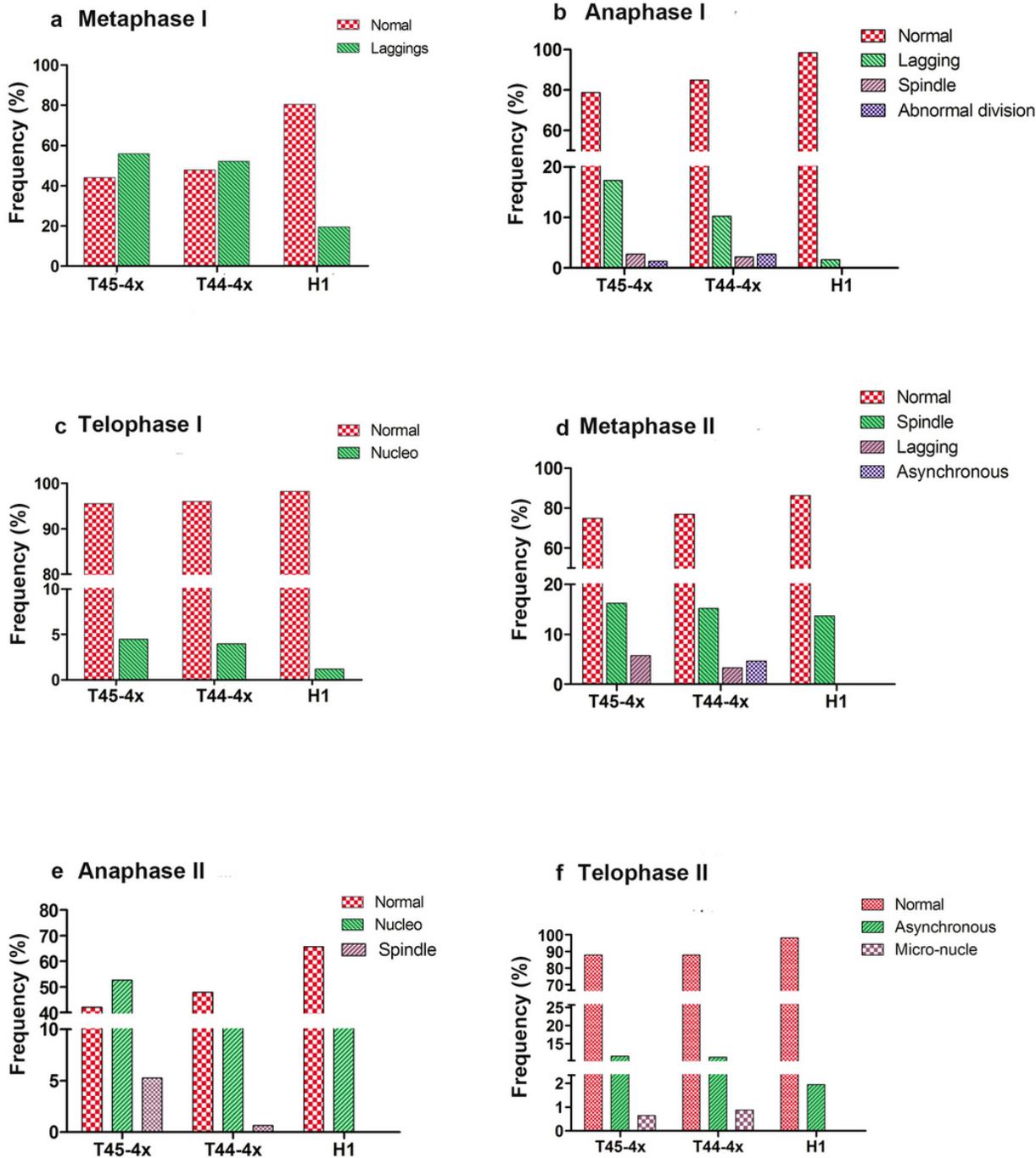
**Figure 1**

Breeding procedure and phenotype of neo-tetraploid rice and its parent. a Breeding procedure of neo-tetraploid rice, Huaduo1 (H1). b Morphologies of whole plant between neo-tetraploid rice, and its two parents. c-e Grain size of H1, and its two parents. f-h pollen grains stained with I2-KI in H1 and its two parents. i-k Comparison of the pollen fertility (i), seed set ratio (j) and plant height (k) between H1 and its two parents.



**Figure 2**

Chromosome behaviors and chromosome configurations in H1 and its parents during PMC meiosis ( $\times 3000$ ). a Zygotene. b Diakinesis. c Diakinesis. d Metaphase I. e Anaphase I. f Telophase I. g Prophase II. h Metaphase II. i Anaphase II. j Telophase II. k Tetrad stage. l Diakinesis, 24 II. m Diakinesis, 12 IV. n Diakinesis, 7 IV+ 10II. o Diakinesis, 9 IV+ 6II. p Diakinesis, univalent (arrow). Bars = 10  $\mu\text{m}$ .



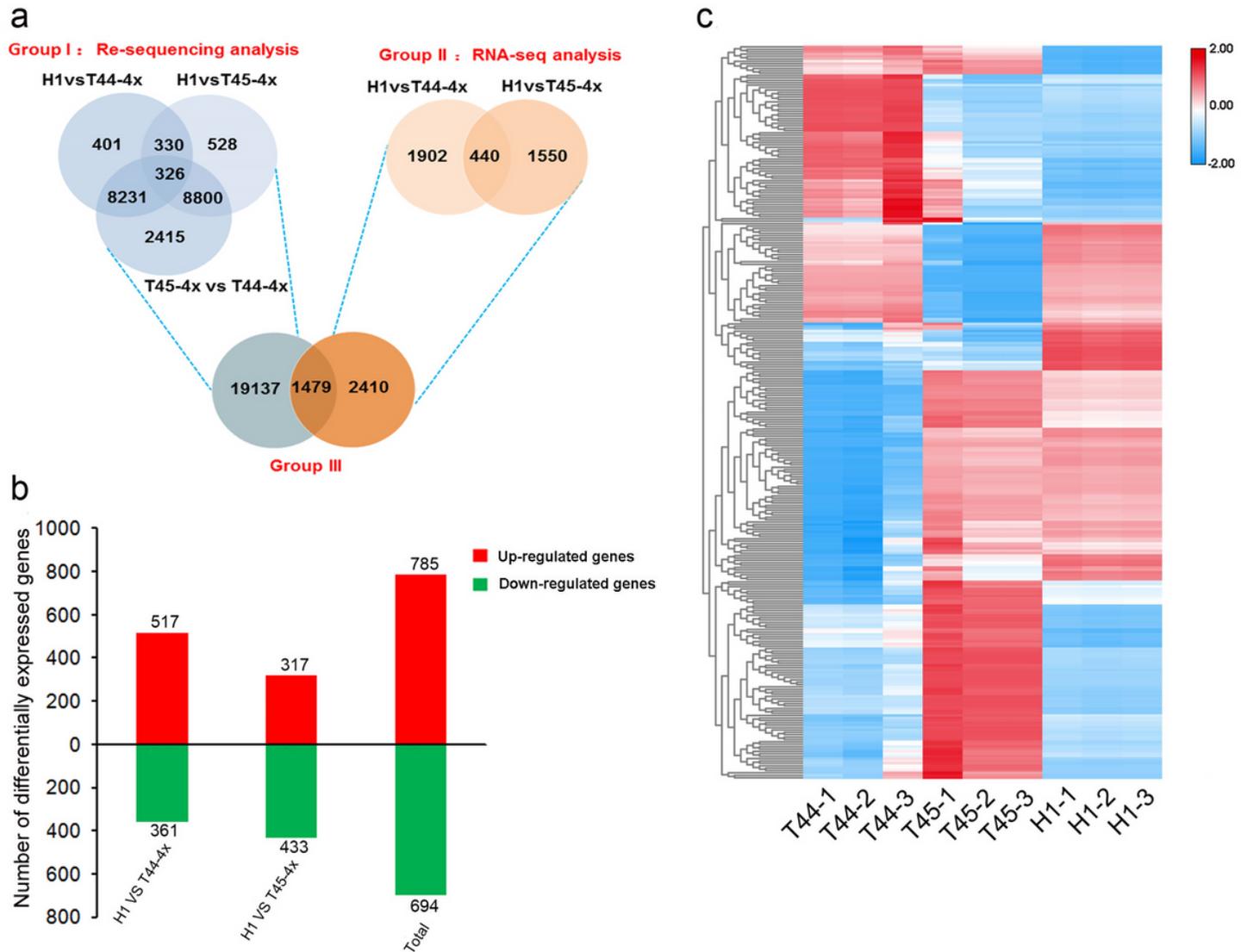
**Figure 3**

Frequency of PMCs in H1 compared with its parents at the meiosis stage. a Frequency of normal cells and main type of abnormal cells at Metaphase I. b Frequency of normal cells and main type of abnormal cells at Anaphase I. c Frequency of normal cells and main type of abnormal cells at Telophase I. d Frequency of normal cells and main type of abnormal cells at Metaphase II. e Frequency of normal cells and main type of abnormal cells at Anaphase II. f Frequency of normal cells and main type of abnormal cells at Telophase II.



**Figure 4**

Number and distribution of SNPs and Indels detected on the rice chromosomes. a Number of SNPs on each rice chromosome derived from T44-4x, T45-4x and Non-parents. b Total number of SNPs detected on each rice chromosome derived from T44-4x, T45-4x and Non-parents.



**Figure 5**

Differentially expressed genes in neo-tetraploid rice detected by Re-sequencing and RNA-sequencing analysis compared to its two parents. a Venn diagram of differentially expressed genes in neo-tetraploid rice detected by Re-sequencing and RNA-sequencing analysis. b Number of differentially expressed genes in neo-tetraploid rice. c Expression patterns of different groups in neo-tetraploid rice and its two parents. Red and green colors indicate up- and down-regulated genes, respectively.

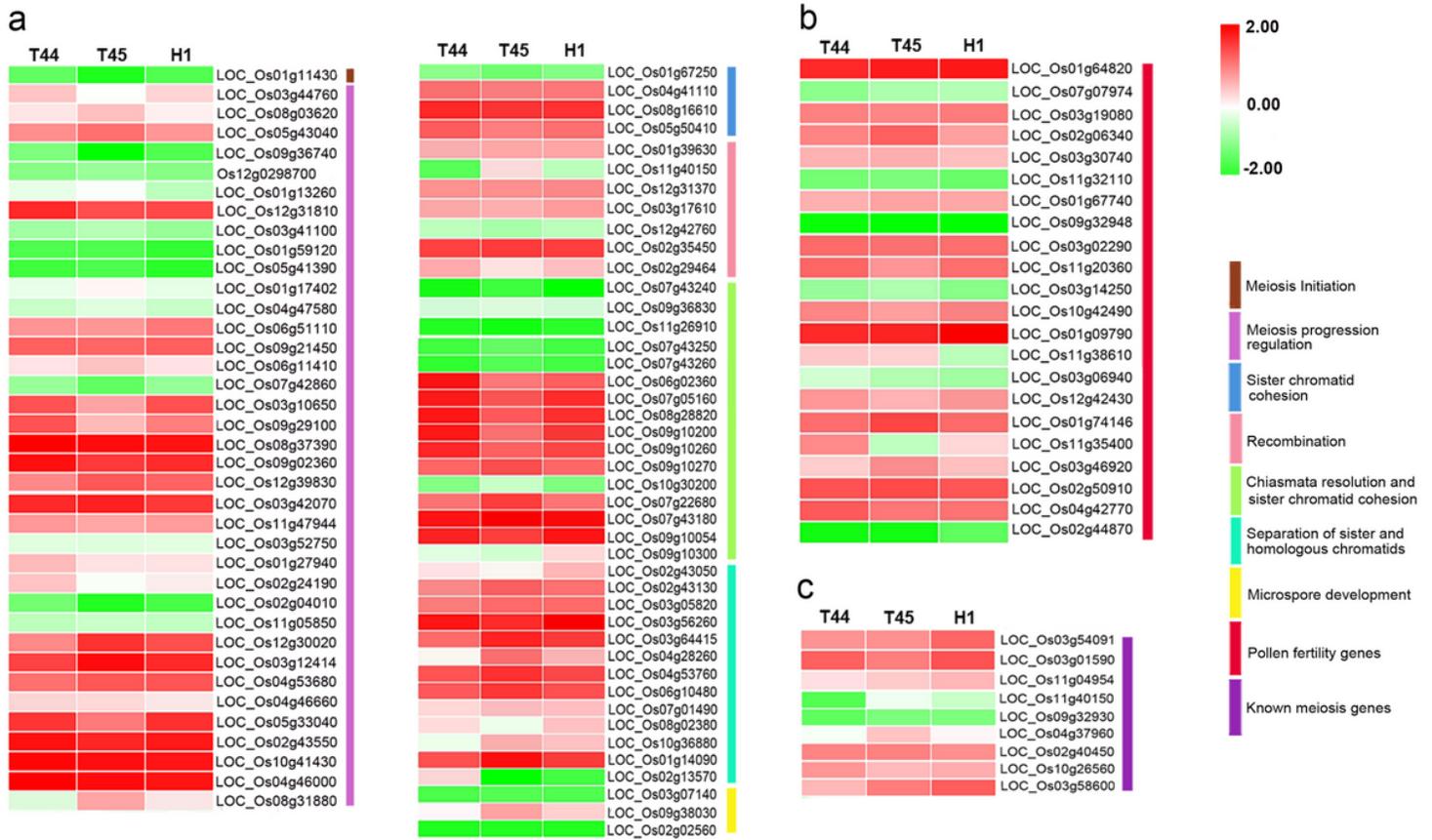
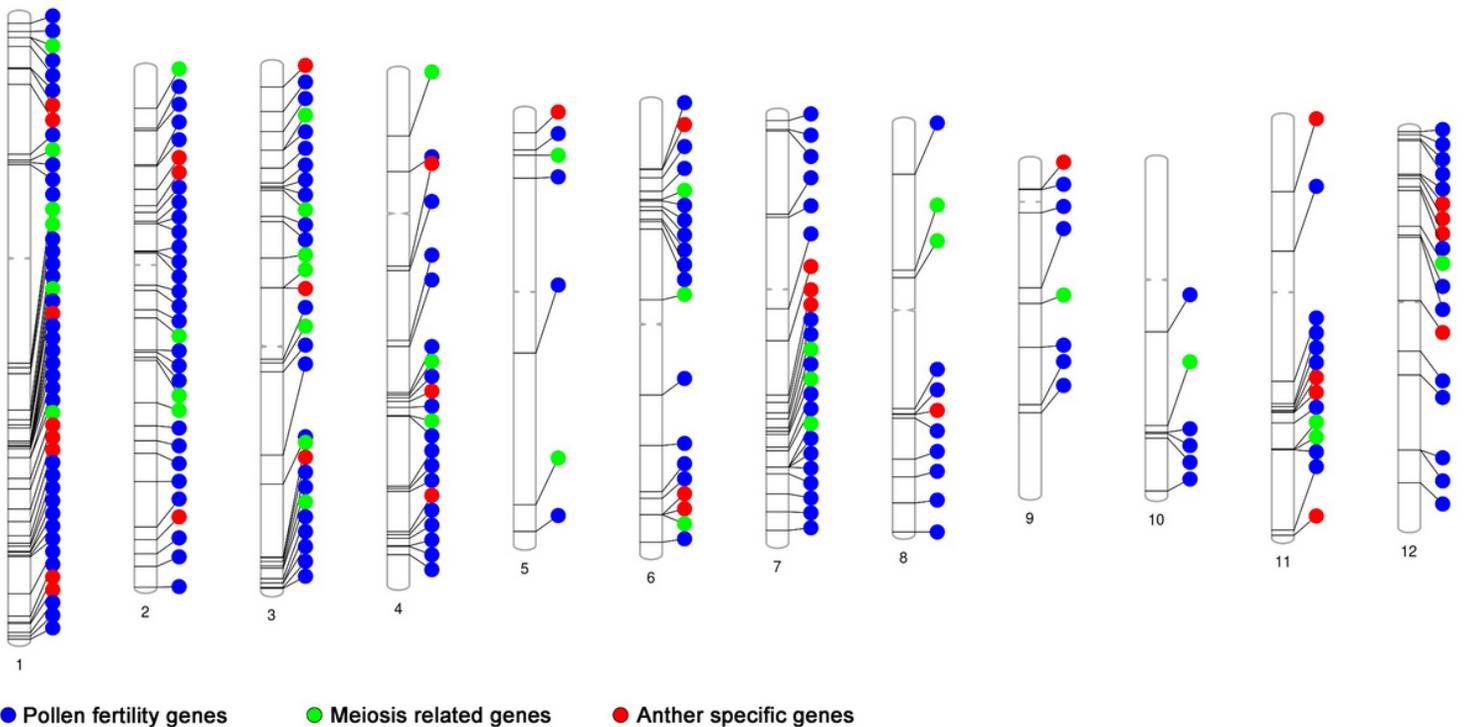


Figure 6

Expression patterns of genes may be involved in meiosis and microspore development. a Expression patterns of meiosis genes. b Expression patterns of the 12 known tapetum-related genes. c Expression patterns of the 12 known pollen fertility genes.



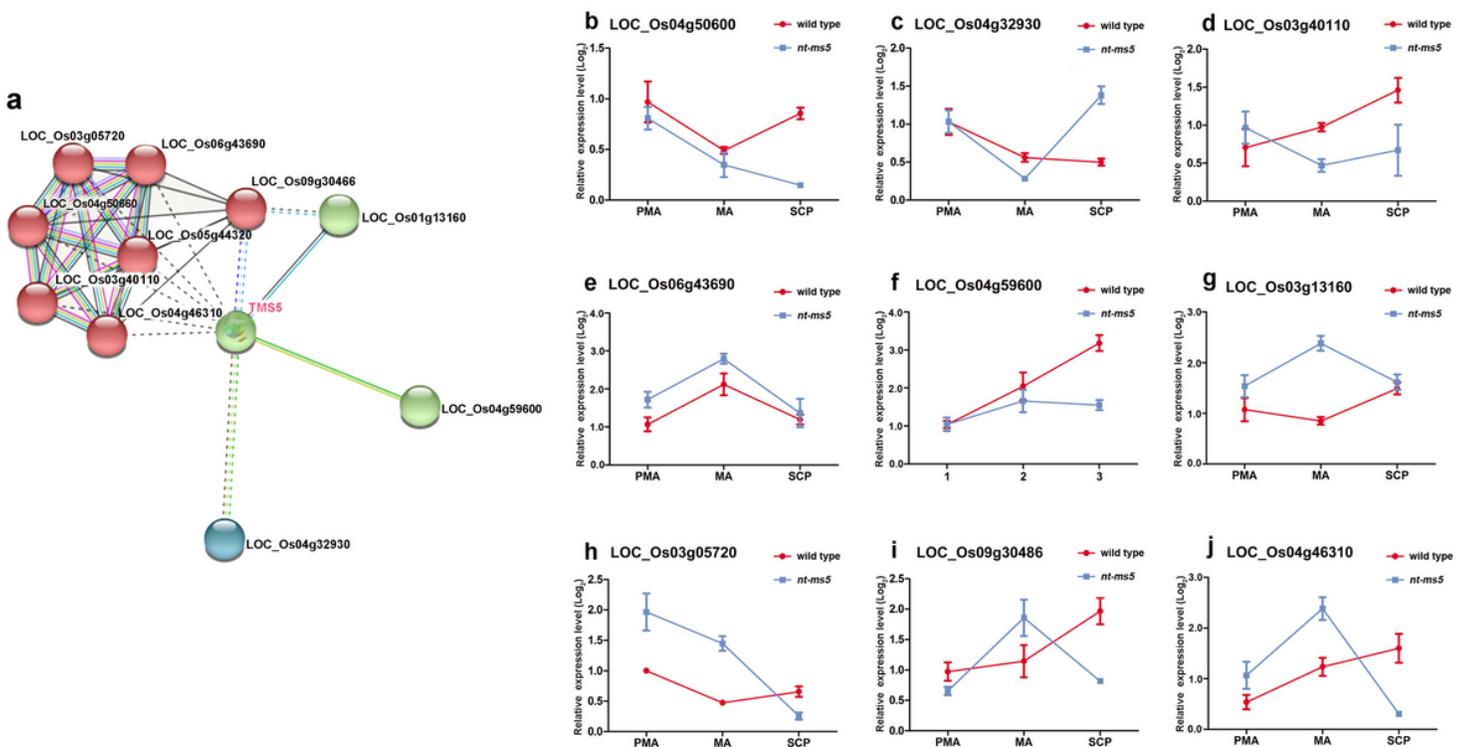
## Figure 7

The distribution of up-regulated genes involved in meiosis process and anther specific in neo-tetraploid rice comparative with its two parents.



## Figure 8

Phenotypic comparison and developing rice anthers between *nt-tms5-1* and its wild type (WT) in neo-tetraploid rice. a and b Floral organs between the wild type and *nt-tms5-1* after removed the lemma. Bars = 1 mm. c and d Anthers between the wild type and *nt-tms5-1* using the WE-CLSM analysis. Bars = 100  $\mu$ m. e and f Comparison of the panicle between the wild type and *nt-tms5-1*. g and h Pollen grains stained with 1% I2-KI solution showing mature pollen grains in WT and typical abortion of mature pollen grains in *nt-tms5-1*. Bars = 100  $\mu$ m. i and j Pollen grains stained with 10 mg/L eosin B solution showing mature pollen grains in WT and typical abortion of mature pollen grains in *nt-tms5-1*. Bars = 50  $\mu$ m. k to p Semi-thin sections of wild type anthers. k meiosis stage; l meiosis stage; m, meiosis stage; n meiosis stage; o late bicellular stage; p mature pollen stage. q to v Semi-thin sections of *nt-tms5-1* anthers. q pre-meiotic interphase; r meiosis stage; s meiosis stage; t single microspore stage; u late bicellular; v mature pollen stage. E, En, M and T indicate the epidermis, endothecium, middle layer and tapetum, respectively. Bars = 50  $\mu$ m.



## Figure 9

Verification of the predicated TMS5 regulation network during the pollen development process in *nt-tms5-1* and its wild type. a Predicted protein-protein interaction network of TMS5 gene. b to j Quantitative real-time PCR (qRT-PCR) confirmation of the regulation network difference between the *nt-tms5-1* and its wild type using the qRT-PCR analysis. b, c, d and f, indicated lower gene expression level in *nt-tms5-1* compared with its wild type. e, g, h, i and j, indicated higher gene expression level in *nt-tms5-1* compared with its wild type. PMA, indicated the pre-meiosis stage. MA, indicated the meiosis stage. SCP, indicated single microspore stage.

## Supplementary Files

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