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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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 440 differentially expressed genes (DEGs) in neo-tetraploid rice compared with its two parents. Of these DEGs, 193 were annotated as pollen fertility-related genes, and 129 (~66.8%) exhibited significant upregulation in neo-tetraploid rice compared with its two parents, including nine cloned genes (TMS9-1 , TMS5 etc.) that were validated by qRT-PCR and had been demonstrated to be pollen fertility-related genes. We further selected TMS9-1 and TMS5 as the candidate gene and analysed its pollen fertility in neo-tetraploid rice using the CRISPR/Cas9 technique. Significant variations have been detected in pollen fertility value, pollen development process and expression level in H1 and its knock out 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 provides 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). S*everal 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; Chen et al. 2019); 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_2

and F_3 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 (Yu et al. 2020). 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 the 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 and its parents, 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 when it crossed with autotetraploid rice (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 1: Table S2). All of the hybrids had a high seed setting (>80%) with gene interactions in pollen sterility loci, *Sa*, *Sb* and *Sc*

(Additional file 1: 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 1: Table S3; Additional file 1: 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 1: Table S3; Additional file 1: 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 1: Table S3; Additional file 1: 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 1: 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 1: 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 1: 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 1: 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 T44-4x, and detected by the comparison between (H1 vs T45-4x) and (T45-4x vs T44-4x). Group II is termed to the genetic variation only from the T45-4x, and detected by the comparison between (H1 vs T44-4x) and (T45-4x vs T44-4x). Group III is termed to the non-parental variations of neo-tetraploid rice (Additional file 1: 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 2: 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 detected on the Chr1, Chr5, Chr6 and Chr12 (Fig. 4).

We further combined these genetic variations with the known 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 1: Table S8a and 8b). To validate this genetic variation data in neo-tetraploid rice, we selected nine pollen fertility genes and used the Sanger sequencing analysis to verify 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 1: Table S9).

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

Transcriptome analysis was further conducted to explore the possible variations of gene expression level associated with high pollen fertility seen in neo-tetraploid rice. We detected the gene expression between neo-tetraploid rice and its parents to identify the possible DEGs of high pollen fertility. From this study, we also divided the DEGs into three groups based on their origin which deriving from two parents or non-parental variations (Fig. 5). Group I is termed to the DEGs in neo-tetraploid rice derived from the T44-4x, and these genes were only differentially expressed between the neo-tetraploid rice and its parent T44-4x (Fig. 5a and 5b; Additional file 1: Table S10a). Group II is termed to the DEGs derived from the T45-4x, and these genes were only differentially expressed between the neo-tetraploid rice and its parent T45-4x (Fig. 5a and 5b; Additional file 1: Table S10b). Group III is termed to common DEGs and these genes were both differentially expressed in neo-tetraploid rice and its two parents, T44-4x and T45-4x (Fig. 5a and 5b; Additional file 1: Table S10c).

Using these comparison groups, we have identified a total of 3896 genes that are differentially expressed (2-fold at P value<0.05) between the neo-tetraploid rice and its parents in meiosis (Fig. 5a). Among these DEGs, 1140 and 767 genes were found to be up- and down-regulated in the Group I, respectively (Fig. 5b; Additional file 1: Table S10a). 647 and 907 genes were found to be up- and down-regulated in the Group II, respectively (Fig. 5b; Additional file 1: Table S10b). Additionally, total 440 genes were detected and found both differentially expressed in neo-tetraploid rice compared with its two parents. Indeed, 255 and 185 genes were found to be up- and down-regulated, respectively (Fig. 5b; Additional file 1: Table S10c). We then focus on these common DEGs which were both differentially expressed in neo-tetraploid rice compared with its two parents. We categorized both the up- and down-regulated 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 common up- and down-regulated DEGs that were differentially expressed between the neo-tetraploid rice and its parents (Additional file 2: Figure S2). The GO enrichment classification suggested that the genes from biological process, cellular component and molecular function categories showed significant variation. In the biological process category, six prominent functional gene classes, including cellular process, transport, transcription, reproductive process, reproduction and pollination, were over-represented in the up-regulated gene classes. Additionally, response to stimulus, response to stress and response to endogenous stimulus were over-represented in the down-regulated gene classes (Additional file 2: Figure S2a). In the cellular component category, three prominent functional gene class, the nucleus, integral to membrane and intrinsic to membrane were over-represented in the up-regulated gene classes, and four prominent functional gene classes, extracellular region, cell wall, external encapsulating structure and thylakoid, were over-represented in the downregulated gene classes (Additional file 2: Figure S2b). In the molecular function category, four prominent functional gene classes, transcription regulator activity, DNA binding, transporter activity and cation binding, were over-represented in the up-regulated gene classes, and four prominent functional gene classes, including catalytic activity, ion binding, oxidoreductase activity and nucleotide binding, were over-represented in the downregulated genes

(Additional file 2: Figure S2c). All of these results indicate that the up-regulated genes were mainly involved in transport, transcription regulator activity-related and reproduction 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 up-regulation in neo-tetraploid rice compared with its two parents

To reveal the cause of higher pollen fertility in neo-tetraploid rice, we focused on the pollen fertility related genes which were commonly differentially expressed and existed genetic variation in neo-tetraploid rice by RNA-sequencing and re-sequencing analysis. We compared our DEGs detected from neo-tetraploid rice and its two parents with the large number of pollen fertility-related genes (Aya et al. 201; Fujita et al. 2010; Deveshwar et al. 2011; Yant et al. 2013; Wright et al. 2015; Wu et al. 2014).

Of these DEGs, 193 genes were annotated as pollen fertility genes when combined with other analysis results. Notably, 129 of the pollen fertility genes were shown to be up-regulated in neo-tetraploid rice compared with its two parents (Fig. 6; Additional file 1: 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 2: Figure S3). Moreover, several pollen fertility genes, such as *LOC_Os03g08790*, *LOC_Os04g37570*, *LOC_Os09g27620*, *LOC_Os09g32025*, *LOC_Os11g37280*, *LOC_Os12g28750*, were detected and up-regulated in neo-tetraploid rice. Among these pollen fertility genes, *TMS9-1* (*LOC_Os09g27620*) is a transcript factor containing a PHD-finger domain that controls pollen sterility under high temperature (Qi et al. 2010). In the present work, we also detected the other two genes, named *CSA* (*LOC_Os01g16810*) and *TMS5* (*LOC_Os02g12290*), were differentially expressed in neo-tetraploid rice compared with its low fertility parent T44-4x. Both of *CSA* and *TMS5* 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 (Zhang et al. 2010). *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 (Zhou et al. 2014).

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_Os02g12290*, *LOC_Os03g08790*, *LOC_Os04g37570*, LOC_Os09g01680, *LOC_Os09g27620*, *LOC_Os09g32025*, *LOC_Os11g37280* and *LOC_Os12g28750*, were consistent with the transcriptome analysis. These results indicated that expression levels of nine genes were consistent with the transcriptome analysis, indicating the reliability and accuracy of RNA-sequencing results (Additional file 2: 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_1 hybrids in autotetraploid rice (Guo et al. 2017; Bei et al. 2019; Ghaleb et al. 2020). To verify the up-regulation of pollen fertility gene 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 *TMS9-1* and *TMS5*, as these genes not only found to be up-regulation in neo-tetraploid rice but also play the important role in hybrid cross and it will provide a possibility to utilize the greater hybrid vigor in polyploidy rice. In this study, the knockout lines of *TMS9-1* and *TMS5* exhibited the obvious pollen sterility compared with their wild type materials, respectively (Fig. 7; Additional file 2: Figure S5; Additional file 2: Figure S6; Additional file 2: Figure S7).

In the present work, we selected *TMS9-1* to conduct the further analysis and obtained at least 20 independently regenerated transgenic lines. The mutant lines were grown in the field, and the T_2 mutants were sequenced. We selected one homozygous mutant, named *nt-tms9-1,* and its sequencing results indicate that it carried the sequence deletion that was predicted to lead to an amino acid change (Additional file 2: Figure S5). We grew the plants in August at an LD and under high temperature conditions to verify their pollen fertility. Both *nt-tms9-1* and its wild type showed marked differences in anther, panicle and pollens (Fig. 7a to 7j). Pollen fertility in *TMS9-1* knock-out lines (*nt-tms9-1*) showed complete pollen sterility compared with its wild type (Fig. 7d and 7j). The statistical analysis results demonstrated that the pollen fertility value of *nt-tms9-1* was much lower than that of its wild type and no pollens could be observed in mature pollen stage (Fig. 7h, Additional file 2: Figure S5c). Moreover, Whole-mount eosin B-staining confocal laser scanning microscopy (WE-CLSM) analysis of anther in *nt-tms9-1* further verified that no pollens were existed in anthers compared with that of the wild type (Fig. 7c, 7d, 7i and 7j).

Anther development was investigated further for *nt-tms9-1* and its wild type. The results indicate that the anther development of *nt-tms9-1* and its wild type were 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 (Fig. 7k to 7v). 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-tms9-1* anthers in the formation of PMCs (Fig. 7k and 7q). During pollen mother cell (PMC) meiosis, dyads and tetrads were normally formed in WT and *nt-tms9-1* (Fig. 7l, 7m, 7r and 7s). Thereafter, the microspores of WT underwent vacuolation and mitosis to form mature pollen with spherical or elliptical shapes (Fig. 7n to 7p). In contrast, the microspores of *nt-tms9-1* degraded further after the late microspore stage and completely disappeared at the mature pollen stage, which resulted in an empty anther locule (Fig. 7t to 7v). All of these results suggest that the lack of *TMS9-1* causes the defects in the microspores and produced no pollens in neo-tetraploid rice during the pollen development.

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 Huaduo1 (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 PMC cells 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 used 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 440 common 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 down-regulated genes that were differentially expressed in neo-tetraploid rice and its parents in this study. The GO enrichment classification indicated that DEGs 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 transport, transcription regulator activity-related and reproduction process.

Meiosis is an important development 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 up-regulated in neo-tetraploid rice when compared with autotetraploid rice. Therefore, we focused on up-regulated DEGs 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 129 pollen fertility genes were detected and found to be up-regulated in neo-tetraploid rice compared with its two parents. Notably, 79 pollen fertility genes were detected in Wu et al. (2014) and Wu et al. (2015), and 37 pollen fertility genes showed a similar tendency as those by Guo et al. (2017). Moreover, these pollen fertility genes in neo-tetraploid rice were found to involve a stronger network based on the predicated protein-protein interaction analysis. These results verify our transcriptome analysis data and indicate that pollen fertility genes play an important role in

neo-tetraploid rice. Notably, three pollen fertility genes, *TMS9-1* (*LOC_Os09g27620*), *TMS5* (*LOC_Os02g12290*) and *CSA* (*LOC_Os01g16810*), are pollen fertility-related genes and also 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 (Zhang et al. 2010). *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 (Zhou et al. 2014). *TMS9-1* (*LOC_Os09g27620*) is a male sterility gene that responsive to 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 few information regarding the effect of knocking out important fertility genes in neo-tetraploid rice（Yu et al. 2020）. In the present work, we detected three environmentally sensitive genic male sterile genes, *TMS9-1* (*LOC_Os09g27620*), *TMS5* (*LOC_Os02g12290*) and *CSA* (*LOC_Os01g16810*), that were up-regulated in neo-tetraploid rice compared with its two parents. We proposed that pollen fertility-related genes may be up-regulated or normally expressed in neo-tetraploid rice compared with those of the autotetraploid rice. Down-regulation of pollen fertility related genes or meiosis related genes were the primary cause of low pollen fertility in autotetraploid rice (Wu et al. 2014; Li et al. 2018). Huaduo1 (H1) exhibited higher fertility, and the seed set ratio can reach to 80% while compared with the autotetraploid rice lines. Therefore, we selected two important gene, *TMS9-1* and *TMS5*, to verify our speculation in neo-tetraploid rice. *TMS9-1* is a male sterility gene that responsive to temperature (Qi et al. 2014). *TMS5* is an important photoperiod- and thermo-sensitive gene that can lead to the TGMS trait through a loss of RNase Z^{S1} function (Zhou et al. 2014; Zhou et al. 2016). Our results indicated that knockout lines of *TMS5* exhibited the pollen sterility in neo-tetraploid rice, and this result was similar as previous analysis (Zhou et al. 2016).

From this study, we also knocked out the *TMS9-1* and obtained its homozygous mutant line, named *nt-tms9-1*. Our results indicated that *nt-tms9-1* exhibited marked differences in anther, panicle and pollen fertility value compared with its wild type. We further performed the cytological analysis and found that pollen fertility value and pollen development process experienced differential variation during the pollen development stage in *nt-tms9-1*. As *TMS9-1* and *TMS5* are important photoperiod- and thermo-sensitive genes, 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 up-regulation 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 129 up-regulated 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 parents (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 in our lab. 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 = (number of filled grains/total number of grains) × 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 × 100%, and MPH = (F_1 − MP)/MP × 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. (2011) 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 HiSeqTM2500 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 Indels analyses, between neo-tetraploid rice and its two parents was performed with GATK software tools. SNPs and Indels 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 1: 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 to 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℃ 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 HiSeqTM2500 by Biomarker Technologies (Beijing, China). Genes with FC≥2 (fold change) and FDR≤0.01 were chosen for the t-test, and genes with P 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 nine candidate genes were selected and used to validate the transcriptome data using the same RNA samples of RNA-sequencing (Additional file 1: Table S12b). Reverse transcription reaction was done 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 (Livak et al. 2001). Rice ubiquitin gene used as an internal control to normalize the expression levels.

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

We used the CRISPR-Cas9 binary vector pC1300-Cas9 to knock out *TMS9-1* and *TMS5* obtained the *nt-tms9-1 and nt-tms5-1* 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-tms9-1* and *nt-tms5-1* mutant line. We extracted the genomic DNA from transformants, and the genomic DNA was sequenced for mutant identification. The PCR products (500-800bp) 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 and WE-CLSM analysis of *nt-tms9-1* and its wild type in neo-tetraploid rice

Samples of *nt-tms9-1* 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. The detailed procedures have been described previously (Li et al. 2018).

WE-CLSM analysis was used to detect the phenotypic variation of H1 and *nt-tms9-1*. 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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Additional Files

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

Additional file 1: Table S2. Seed set ratio of hybrids with genetic interactions at *Sa*, *Sb* and *Sc* loci.

Additional file 1: Table S3. Frequency of abnormal chromosome behaviors in neo-tetraploid rice and its parents during the Meiosis I.

Additional file 1: Table S4. Frequency of abnormal chromosome behaviors in neo-tetraploid rice and its parents during the Meiosis II.

Additional file 1: Table S5. Meiotic chromosome configurations in neo-tetraploid rice and its parents.

Additional file 1: Table S6. Summary of the re-sequencing data in neo-tetraploid rice and its two parents.

Additional file 1: Table S7. Summary of SNP and InDels in neo-tetraploid rice compared with its two parents.

Additional file 1: Table S8a. Summary of the pollen fertility related QTLs in neo-tetraploid rice compared with its two parents based on re-sequencing analysis.

Additional file 1: Table S8b. Summary of the pollen fertility related genes in neo-tetraploid rice compared with its two parents based on re-sequencing analysis.

Additional file 1: Table S9. Validation of re-sequencing variations between the neo-tetraploid rice and its parents by Sanger sequencing.

Additional file 1: Table S10a. Differentially expressed genes in neo-tetraploid rice derived from T44-4x.

Additional file 1: Table S10b. Differentially expressed genes in neo-tetraploid rice derived from T45-4x.

Additional file 1: Table S10c. Common differentially expressed genes in neo-tetraploid rice compared with its parents.

Additional file 1: Table S11. Functional pollen fertility related genes in neo-tetraploid rice compared with its two parents.

Additional file 1: Table S12a. List of primers used for re-sequencing analysis.

Additional file 1: Table S12b. List of primers used for qRT-PCR analysis.

Additional file 2: Figure S1. Chromosome-wide counts distribution of SNP and Indels per 100kb between H1 and its two parents.

Additional file 2: Figure S2. GO analysis of common differentially expressed genes in neo-tetraploid rice comparative its two parents.

Additional file 2: Figure S3. Predicted protein-protein interaction network of pollen fertility genes in neo-tetraploid rice compared with its parents.

Additional file 2: Figure S4. Comparison of the $\log_2$ (FC) of nine selected genes using qRT-PCR analysis.

Additional file 2: Figure S5. Mutations of *TMS9-1* target sites and its expression level in Huaduo1 and *nt-tms9-1*.

Additional file 2: Figure S6. Mutations of *TMS5* target sites and its expression level in Huaduo1 and *nt-tms5*.

Additional file 2: Figure S7. Phenotypic comparison and developing rice anthers between *nt-tms5* and its wild type in neo-tetraploid rice.

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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.

Note: 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.

Figures

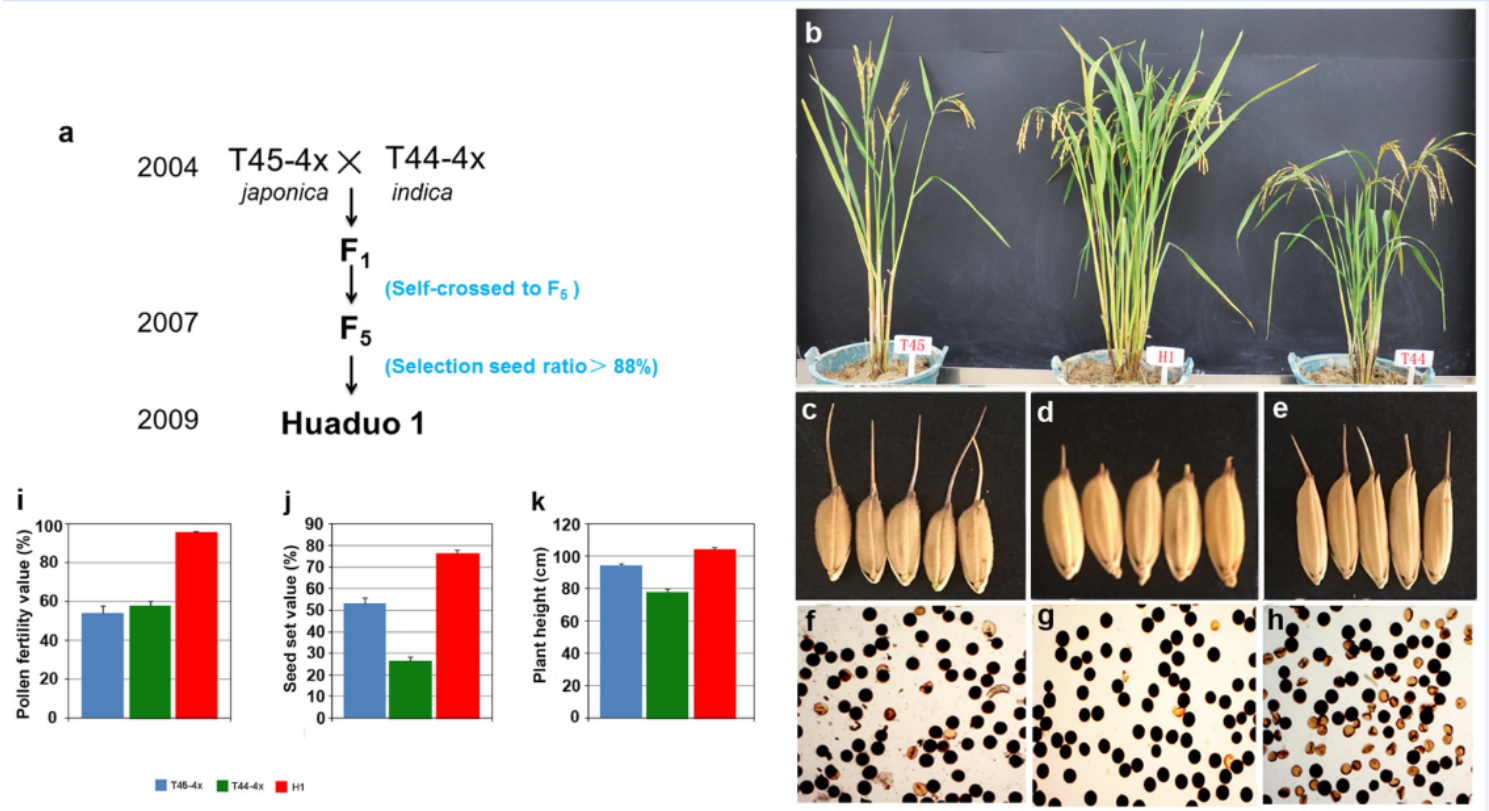

Figure 1

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.

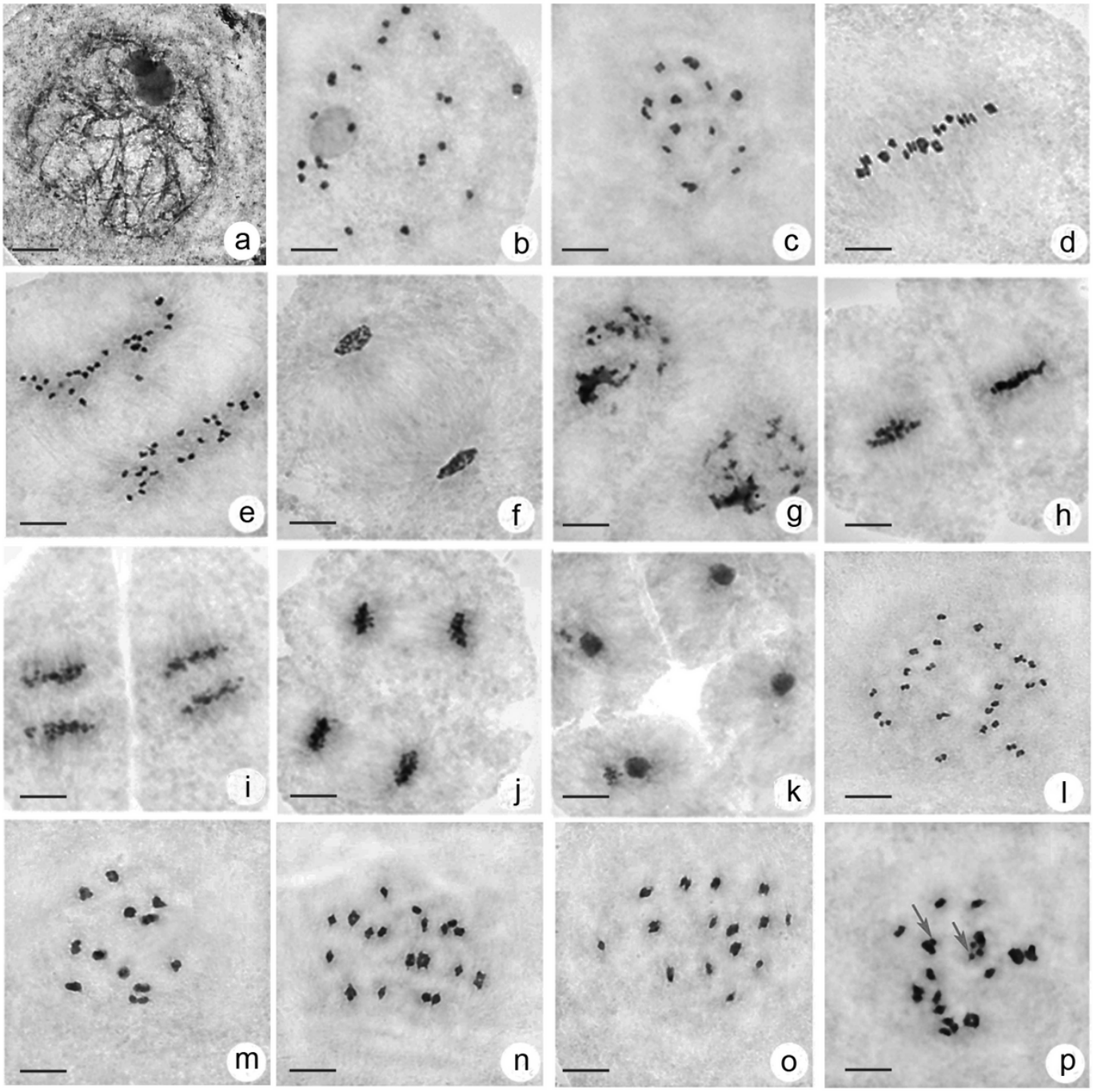

Figure 2

Chromosome behaviors and chromosome configurations in H1 and its parents during PMC meiosis (×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 μm.

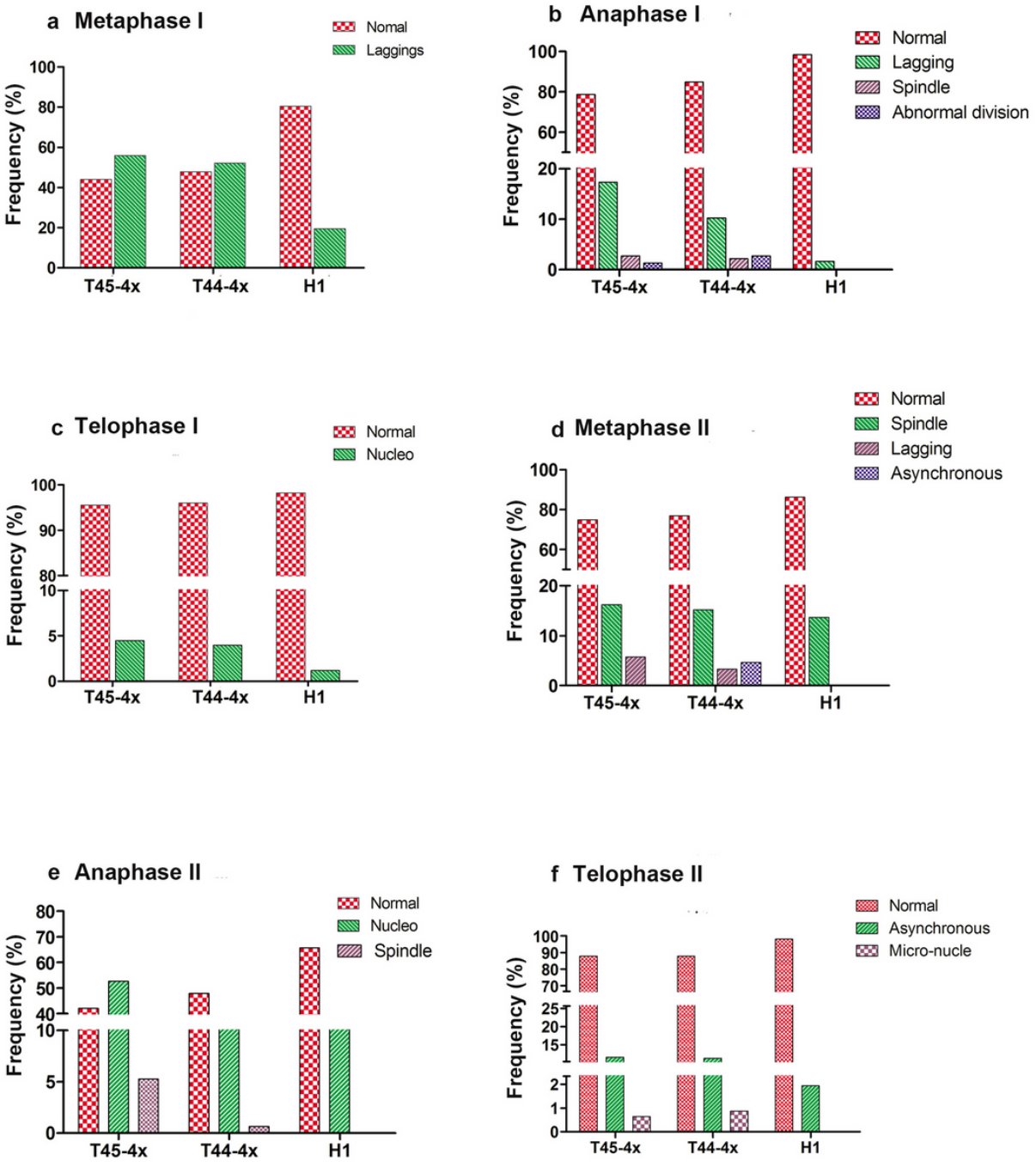

Figure 3

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.

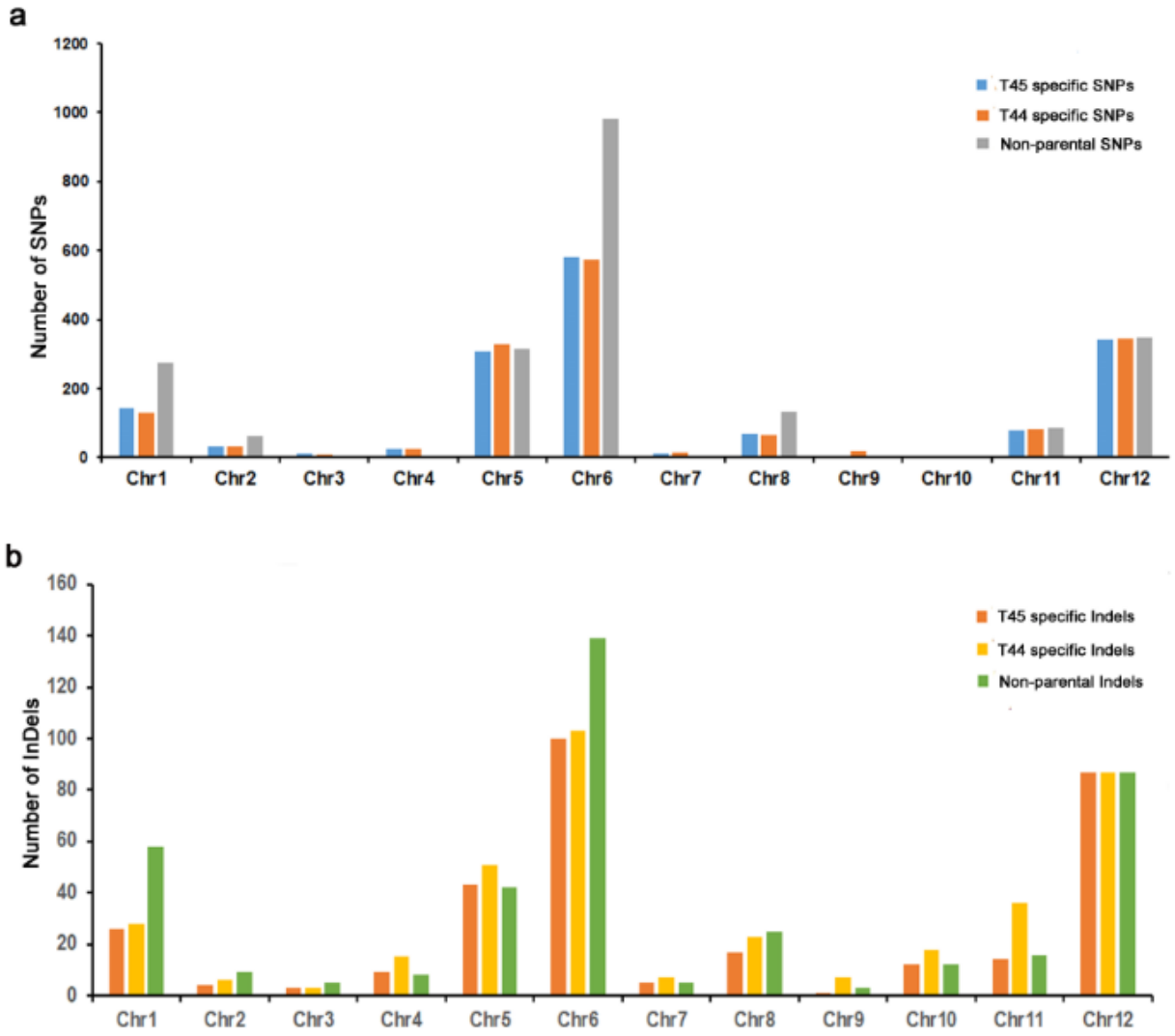

Figure 4

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.

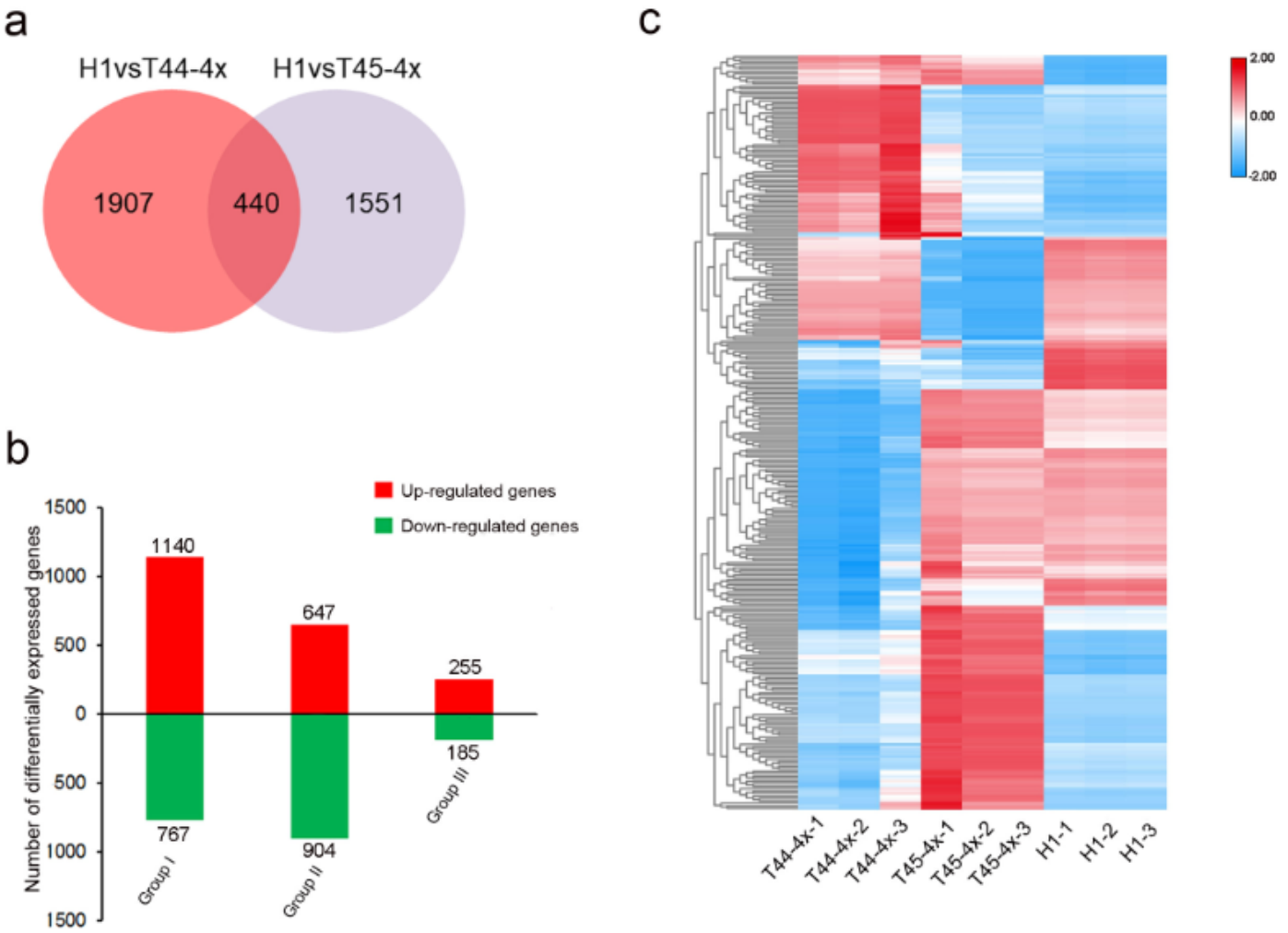

Figure 5

Differentially expressed genes in neo-tetraploid rice detected by RNA-sequencing analysis compared to its two parents. a Venn diagram of differentially expressed genes in neo-tetraploid rice detected by RNA-sequencing analysis. b Number of differentially expressed genes in neo-tetraploid rice compared its parents. Group I is termed to the DEGs of neo-tetraploid rice derived from T44-4x; Group II is termed to the DEGs of neo-tetraploid rice derived from the T45-4x; Group III is termed to common were both differentially expressed in neo-tetraploid rice and its two parents. c Expression patterns of common DEGs in neo-tetraploid rice and its two parents. Red and blue colors indicate up- and down-regulated differentially expressed genes, respectively.

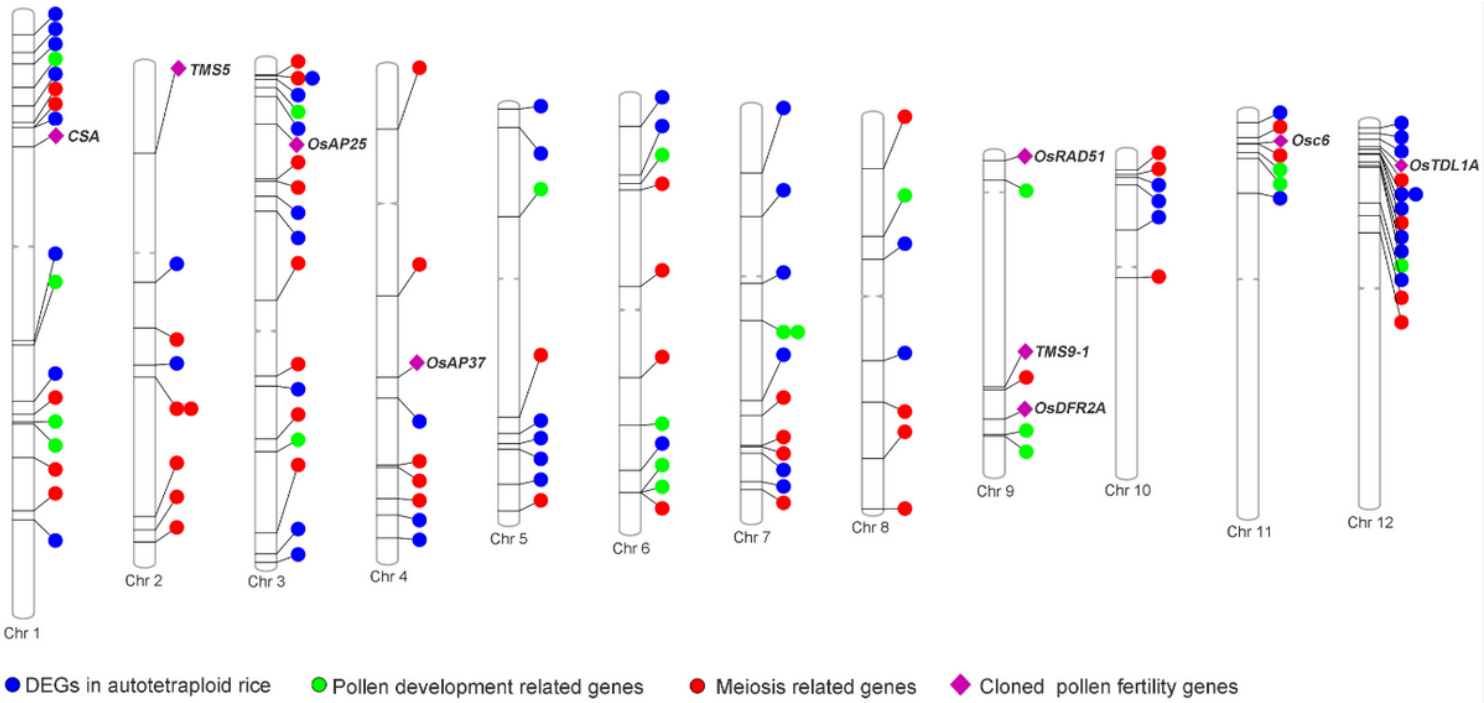

Figure 6

The distribution of up-regulated genes involved in meiosis process and pollen development process in neo-tetraploid rice comparative with its two parents. DEGs in autotetraploid rice: DEGs detected in pollen development process of autotetraploid rice. Pollen fertility genes: DEGs involved in pollen development process. Meiosis related genes: DEGs involved in meiosis process. Cloned pollen fertility genes: the known pollen fertility DEGs.

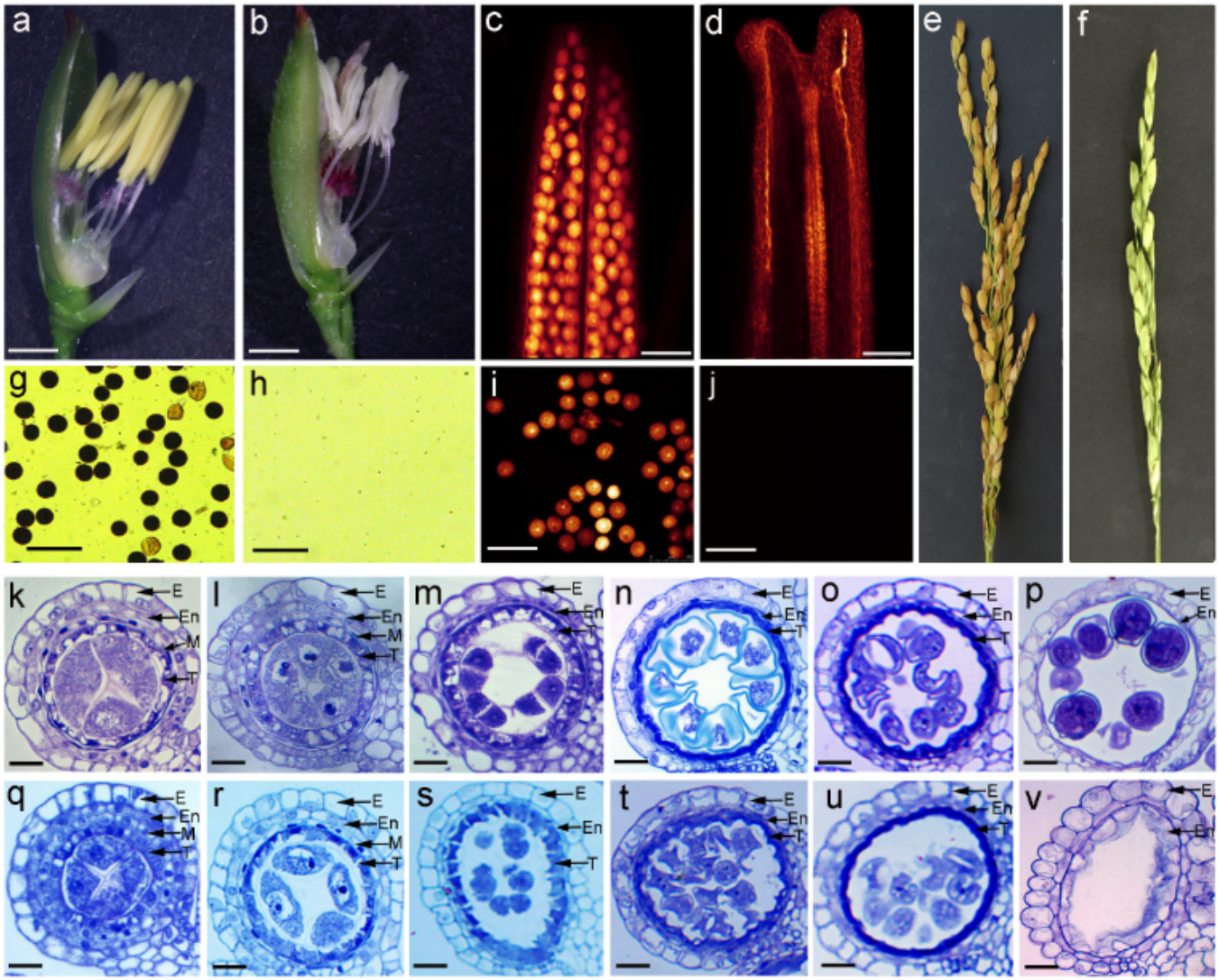

Figure 7

Phenotypic comparison and developing rice anthers between nt-tms9-1 and its wild type (WT) in neo-tetraploid rice. a and b Floral organs between the wild type and nt-tms9-1 after removed the lemma. Bars = 1 mm. c and d Anthers between the wild type and nt-tms9-1 using the WE-CLSM analysis. Bars = 100 μm. e and f Comparison of the panicle between the wild type and nt-tms9-1. g and h Pollen grains stained with 1% I2-KI solution showing mature pollen grains in WT and abortion of mature pollen grains in nt-tms9-1. Bars = 100 μm. i and j Pollen grains stained with 10 mg/L eosin B solution showing mature pollen grains in WT and abortion of mature pollen grains in nt-tms9-1. Bars = 50 μm. k to p Semi-thin sections of wild type anthers. k meiosis stage I; l meiosis stage I; m meiosis stage II; n single microspore stage; o late bicellular stage; p mature pollen stage. q to v Semi-thin sections of nt-tms9-1 anthers. q pre-meiotic interphase; r meiosis stage I; s meiosis stage II; 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 μm.

Supplementary Files

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- Supplementalfiles1tables.xlsx
- Supplementalfile2figures.pptx