Plant Physiology and Biochemistry 141 (2019) 73–82

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Cytological and transcriptome analysis reveal that interaction at *Sb* pollen sterility locus cause down-regulation of important meiosis-related genes associated with high pollen sterility in autotetraploid rice hybrids

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ARTICLE INFO

Keywords:
Autotetraploid rice
Meiosis
Pollen sterility
Polyploidy
Transcriptome analysis

ABSTRACT

Polyploidy could increase the interactions of pollen sterility loci and *Sb* locus interaction cause higher pollen abortion than other loci. Therefore, we focused on the interaction at *Sb* pollen sterility locus in autotetraploid rice compared to diploid rice hybrid using the near-isogenic lines in the present study. Cytological observations indicated that interaction at *Sb* locus cause high pollen sterility (69.9%) and abnormal chromosome behavior (37.02%) at Metaphase II in autotetraploid rice hybrid. A total of 139 meiosis-related or meiosis stage-specific genes were detected in the autotetraploid rice hybrid harboring interaction at *Sb* locus and 27 of these meiosis-related or specific genes displayed significant down-regulation, including four pollen fertility related genes (*Rad51*, *XRI1*, *PSS1* and *MIL1*). These results revealed a stronger interaction at *Sb* pollen sterility locus than other loci, which cause down-regulation of many important meiosis-related genes that were associated with higher pollen sterility in autotetraploid rice hybrids.

1. Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population, and to ensure the food security of world it is of utmost importance to increase rice yield. Autotetraploid rice is a useful germplasm derived from diploid rice by chromosome doubling. Inter-subspecific autotetraploid rice hybrids exhibited certain biological vigor and heterosis compared to diploid rice (Shahid et al., 2011, 2012; Wu et al., 2013). Several agronomic traits in autotetraploid rice, such as the large grain size, high 1000-grain weight, strong stem and long panicles, have been detected and showed a great advantage compared with their corresponding diploid rice (Shahid et al., 2013a; Wu et al., 2013). However, low pollen fertility is the major hindrance in the utilization of autotetraploid rice (Shahid et al., 2010; He et al., 2011a, b; Wu et al., 2015). Our previous research had demonstrated that multi-allelic interaction of three pollen sterility loci, i.e. *Sa*, *Sb* and *Sc* pollen sterility loci, was the critical factor leading to pollen sterility, and polyploidy could enhance the multi-allelic interaction at three loci that lead to very low pollen fertility in autotetraploid rice hybrids (He et al., 2011a, b; Wu et al., 2015, 2017). *Sa* and *Sc* pollen sterility loci had been cloned and functionally studied in diploid rice hybrids, in which *Sa* consists of two adjacent genes that encode a SUMOE3 ligase-like protein and an F-box protein (Long et al., 2008), and *Sc* harboring structural changes and copy number variation lead to the *indica-japonica* hybrid male sterility (Shen et al., 2017). Among three loci, pollen fertility of diploid hybrid harboring interaction at *Sb* pollen sterility locus was much lower than other single pollen sterility locus (i.e., *Sa* or *Sc*) (Zhang et al., 2006; Shahid et al., 2013b). Our lab also found that the autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus exhibited higher pollen sterility than other loci (Zhao et al., 2006). Therefore, it is of great interest to study pollen sterility effect caused by the interaction at *Sb* pollen sterility locus in autotetraploid rice hybrids.

Transcriptome analysis play important role in understanding the genetic regulation of anther and pollen development. Large numbers of gene expression datasets have been publicly available on Rice XPro

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https://doi.org/10.1016/j.plaphy.2019.05.019
Received 1 April 2019; Received in revised form 9 May 2019; Accepted 18 May 2019
Available online 21 May 2019
0981-9428/

(Rice Expression Profile Database), TIGR (Rice Genome Annotation Project Database), and GEO (Gene Expression Omnibus Database) (Ouyang et al., 2007; Sato et al., 2013). Reliable pollen development network and novel pollen development genes could be detected using these databases. For example, a recent publication of transcriptome analysis revealed 57 rice anther tissue microarray data sets for the identification of novel genes during plant anther and pollen development (Lin et al., 2017). Moreover, several studies have been focused on the pollen development process in autotetraploid rice. Guo et al. (2017) revealed significant variations in neo-tetraploid rice and its two parents, and 42 meiosis stage-specific genes or meiosis-related genes were detected by transcriptome analysis. A total of 75 meiosis-related genes displayed differential expressions in T449 compared to its diploid rice during the meiosis stage (Chen et al., 2018).

Till now, little is known about the cytological mechanism of *Sb* pollen sterility locus in autotetraploid compared to the diploid rice hybrids. Therefore, we developed autotetraploid and diploid rice hybrids, which were heterozygous at *Sb* pollen sterility locus, to evaluate the pollen sterility effect caused by the interaction at *Sb* pollen sterility locus using the near-isogenic lines in the present study. We conducted cytological and transcriptome analyses to analyze the pollen sterility effect caused by the interaction at *Sb* pollen sterility locus in autotetraploid rice hybrids. The specific objectives of this study were: (i) to evaluate the effect of *Sb* pollen sterility locus in autotetraploid rice hybrids; (ii) to identify the differentially expressed genes associated with the interaction effect of *Sb* pollen sterility locus in autotetraploid rice hybrid; (iii) to reveal the primary cause of higher pollen sterility in autotetraploid rice hybrids. Our analysis will provide a comprehensive evaluation of pollen sterility effect of *Sb* pollen sterility locus caused by the interaction in autotetraploid rice hybrids.

2. Materials and methods

2.1. 1 Plant material

Total six hybrids, including three autotetraploid rice hybrids and their corresponding diploid rice hybrids that were heterozygous (S^iS^j) at *Sa*, *Sb* and *Sc* pollen sterility locus, were used in this study. Taichung65–4x and its diploid rice (Taichung65–2x) were used as control to compare with the autotetraploid and diploid rice hybrids. Genotypes of all hybrids and their parents are shown in Table 1. All of these materials were planted under natural conditions at the experimental farm of South China Agricultural University (SCAU) and standard practices were done according to the recommendations of area.

2.2. Pollen fertility observation

Pollen fertility of diploid and autotetraploid rice hybrids was observed according to our previous study with minor modifications (Shahid et al., 2013b). The spikelets were randomly selected from the middle-upper part of panicle and fixed in Carnoy solution for 24 h, and then kept in 70% ethanol. Six anthers from each spikelet were stained in 1% (wt/vol) iodine potassium iodide (I_2-KI) solution on a glass slide to evaluate the pollen fertility. More than 1000 pollen grains were calculated for the pollen fertility under a microscope (Motic BA200). The types of normal and abnormal pollens in diploid and autotetraploid rice hybrids were calculated according to Shahid et al. (2013b).

2.3. Chromosome behavior observation

Chromosome behavior during meiosis was observed according to our previous study with minor modifications (Wu et al., 2014). Inflorescences were collected at meiosis stage and fixed in Carnoy solution (ethanol: acetic acid, 3:1 v/v) for at least 24 h and washed using 95% and 80% ethanol about 30 min each. Finally, samples were washed and kept in 70% ethanol at 4 °C until observation. The procedures of meiosis chromosome behavior observation and meiotic stage's division were according to He et al. (2011a) and Wu et al. (2014).

2.4. Microsporogenesis observation

Microsporogenesis observation was conducted according to our previous study (Wu et al., 2014). The inflorescences in microsporogenesis were collected and kept in petri dish with a moist paper. Before observing under the Leica SPE laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany), anthers were removed from the floret and squashed with the forceps onto the glass slide, and then a small drop of 10 mg/L eosin B ($C_{20}H_6N_2O_9Br_2Na_2$, FW 624.1, a tissue stain for cell granules and nucleoli) solution (dissolved in 4% sucrose) was added, which was covered by a slide cover and observed after 10 min. Excitation wave length was 543 nm, and emission light was detected between 550 and 630 nm (Wu et al., 2014).

2.5. Sample preparation, RNA extraction and microarray analysis

Anthers in Prophase I stage were confirmed by 4',6-diamidino-2-phenylindole (DAPI) staining through a fluorescence microscope (Supplementary Fig. S1). Three biological replicates of autotetraploid rice hybrids and diploid rice hybrids were prepared and stored at −80 °C until RNA extraction. To identify the differentially expressed genes associated with the interaction effect at *Sb* pollen sterility locus, Affymetrix Gene Chip Rice Genome Arrays were used for transcriptome study. Microarray experiments were performed according to Affymetrix protocols (Wu et al., 2015).

Genes with FC ≥ 2 (fold change) or FC ≤ 0.5 (fold change) were chosen for the *t*-test, and genes with P values < 0.05 were chosen for further analysis. After the selection of differentially expressed genes, cluster analysis and GO enrichment analysis were conducted using the Cluster 3.0 software and agriGO (Du et al., 2010). Specific differentially expressed genes at each pollen sterility locus were detected by Venn tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html/). Predicted protein-protein interaction was done by String database (http://string-

Table 1
Genotypes of parents and hybrids at *S-a*, *S-b*, and *S-c* pollen sterility loci.

Material name	Interaction locus	Genotype			Number of pollens	Pollen fertility (% ± SE)
		Sa	*Sb*	*Sc*		
E1-2x	–	Sa^jSa^j	Sb^jSb^j	Sc^jSc^j	1569	96.32 ± 0.01
E1-2x × E5-2x	*Sa*	Sa^iSa^j	Sb^jSb^j	Sc^jSc^j	2617	51.50 ± 0.24
E1-2x × E2-2x	*Sb*	Sa^jSa^j	Sb^iSb^j	Sc^jSc^j	2668	42.95 ± 0.08
E1-2x × E4-2x	*Sc*	Sa^jSa^j	Sb^jSb^j	Sc^iSc^j	1942	48.77 ± 1.26
E1-4x	–	$Sa^jSa^jSa^jSa^j$	$Sb^jSb^jSb^jSb^j$	$Sc^jSc^jSc^jSc^j$	1784	65.36 ± 0.18
E1-4x × E5-4x	*Sa*	$Sa^iSa^jSa^jSa^j$	$Sb^jSb^jSb^jSb^j$	$Sc^jSc^jSc^jSc^j$	1609	50.83 ± 0.32
E1-4x × E2-4x	*Sb*	$Sa^jSa^jSa^jSa^j$	$Sb^iSb^jSb^jSb^j$	$Sc^jSc^jSc^jSc^j$	1415	30.10 ± 0.14
E1-4x × E4-4x	*Sc*	$Sa^jSa^jSa^jSa^j$	$Sb^jSb^jSb^jSb^j$	$Sc^iSc^jSc^jSc^j$	1382	47.91 ± 0.76

Note: E1 represents Taichung 65, while E2, E4 and E5 represent near-isogenic lines of Taichung 65; 2x and 4x represent diploid and autotetraploid rice.

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2.6. qRT-PCR analysis

The expression patterns of candidate genes were validated by qRT-PCR analysis. Primers were designed using the Primer Premier 5.0 and Oligo 7.0 software (Supplementary Table S1). The qRT-PCR experiments were performed on the Lightcycler 480 system (Roche) using the Advanced SYBR Green Supermix Kit (Bio-RAD). The cycling conditions were 95 °C for 30s, followed by 40 cycles of 95 °C denaturation for 5s and 58 °C annealing and extension for 20s. The rice ubiquitin gene was used as an internal control to normalize the level of expression. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001). All qRT-PCR data had three biological replicates.

3. Results

3.1. Allelic interaction at Sb pollen sterility locus cause low pollen fertility in autotetraploid rice hybrid

To compare the allelic interaction effect at *Sb* pollen sterility locus in autotetraploid rice hybrid, we estimated the pollen fertility of six hybrids and their parents (Table 1). In this study, three diploid rice F_1 hybrids, harboring interactions at three single pollen sterility locus (*Sa*, *Sb* and *Sc*), showed lower pollen fertility than their parents (Fig. 1). Autotetraploid rice F_1 hybrids, harboring allelic interaction at different pollen sterility loci (*Sa*, *Sb* and *Sc*), also exhibited lower pollen fertility than their parents, but it was lower than corresponding diploid rice hybrids (Table 1). Notably, pollen fertility of autotetraploid rice hybrid (E1-4x × E2-4x) was the lowest and it was only 30.10% (Table 1). These results demonstrated that autotetraploid rice hybrid, which has the interaction at *Sb* pollen sterility locus, exhibited higher pollen sterility in autotetraploid rice hybrid than other hybrids.

3.2. Allelic interaction at Sb pollen sterility locus cause higher pollen abortion in autotetraploid rice hybrid

To evaluate the pollen sterility effect caused by the allelic interaction at *Sb* pollen sterility locus in autotetraploid rice and diploid rice hybrids, WE-CLSM (whole-mount eosin B-staining confocal laser scanning microscopy) and chromosome behavior observations were conducted (Fig. 2). Here, microsporogenesis undergo two distinct meiotic divisions, including Meiosis I and Meiosis II in diploid rice hybrid. Two types of abnormalities, including spindle abnormalities and cell degeneration, were observed when it entered into the Metaphase II stage (Fig. 2A–E). By contrast, abnormal cells were found at Prophase II, and higher percentage of spindle abnormalities and cell degeneration were observed after the cells moved into the Metaphase II and Tetrad stage in autotetraploid rice hybrids. For example, many kinds of abnormalities, including spindle abnormalities and cell degeneration at Metaphase II and Tetrad stage, were observed in the hybrid harboring *Sb* pollen sterility locus interaction (Fig. 2F–I).

Pollen mother cells (PMCs) meiosis behaviors were also conducted to evaluate the *Sb* pollen sterility effect compared to other single pollen sterility locus (*Sa* and *Sc*). Total five important stages, including Metaphase I, Anaphase I, Metaphase II, Anaphase II and Telophase II, were observed and frequencies of abnormal cells are summarized in Table 2 and Table 3. Notably, abnormal percentage of PMC cells harboring interaction at *Sb* pollen sterility locus during Metaphase II and Anaphase II depicted significant differences between autotetraploid rice hybrids and diploid rice hybrids and other single pollen sterility locus hybrids.

In Metaphase I, 2.6% of cells displayed chromosome lagging in autotetraploid rice hybrid, and the frequencies of abnormal cells in diploid and autotetraploid rice hybrids were 0.21% and 2.6%, respectively (Table 2). Chromosome straggling and abnormal spindles were the primary abnormality types, and higher percentage of abnormalities (3.25%) was observed in autotetraploid rice hybrid with the interaction at *Sb* pollen sterility locus than its diploid rice hybrid at Anaphase I (Table 2). In Metaphase II, chromosome lagging, asynchronous cell division and abnormal spindles were the major abnormalities, and total abnormality percentage was more than 30% in autotetraploid rice

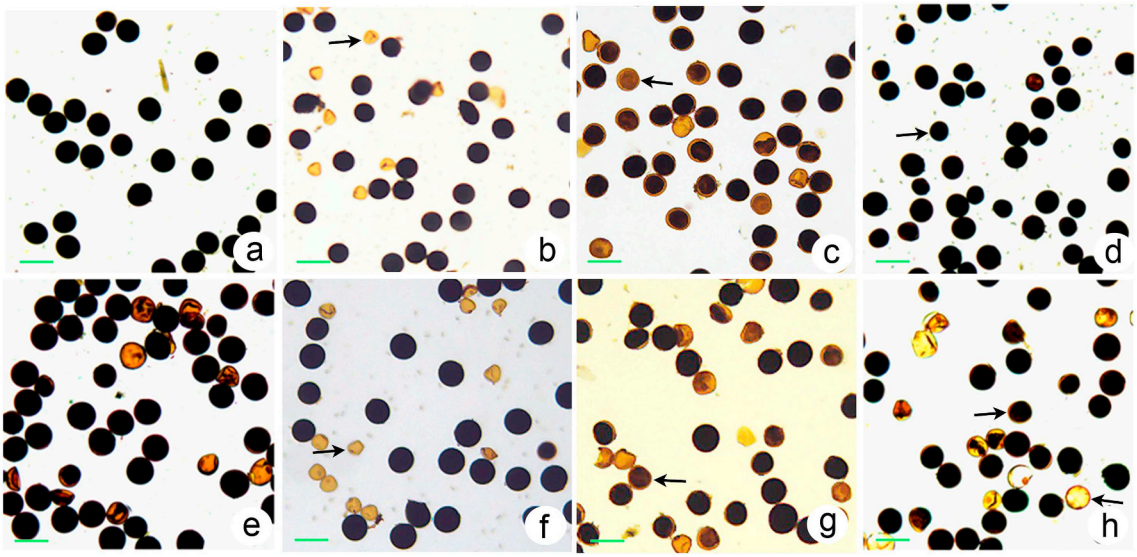


Fig. 1. Pollen fertility of hybrid and parents in diploid and autotetraploid rice. (A) and (E) indicated the pollen phenotypes of Taichung65–2x and Taichung65–4x, respectively. (B) and (F) indicated the pollen phenotypes of diploid and autotetraploid rice hybrid with interaction at *Sa* locus, respectively; Arrows indicated the typical abortive pollen grains (irregular). (C) and (G) indicated the pollen phenotypes of diploid and autotetraploid rice hybrid with interaction at *Sb* locus, respectively; Arrows indicated the stained abortive pollen grains (round and un-stained). (D) and (H) indicated the pollen phenotypes of diploid and autotetraploid rice hybrid with interaction at *Sc* locus, respectively; Arrows indicated the spherical abortive or small pollen grains (small or empty abortive pollen grains). Bars = 10 μm.

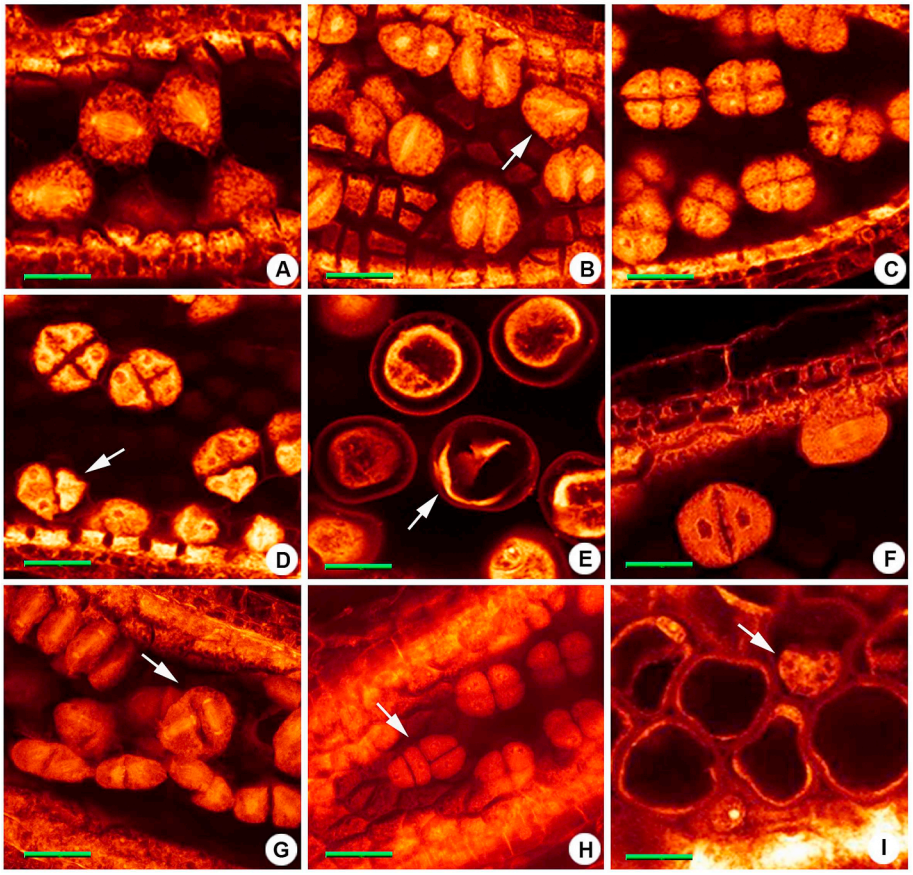


Fig. 2. Pollen abortion caused by the interaction of *Sb* pollen sterility locus in autotetraploid rice hybrid compared with diploid hybrid. (A–E) Abnormal pollen development in diploid rice hybrid harboring interaction at *Sb* pollen sterility locus. (A) Telophase I, normal cells in this stage. (B) Metaphase II, abnormal spindles. (C) Tetrad stage, normal cells in this stage. (D) Tetrad stage, two microspores degradation. (E) Late single microspore stage, cell degeneration. (F–I) Abnormal pollen development in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus. (F) Prophase II, spindle abnormalities. (G) Metaphase II, spindle abnormalities. (H) Tetrad stage, T type tetrad. (I) Late single microspore stage, cell degeneration. Bars = 40 μm.

hybrid under the interaction of *Sb* pollen sterility locus (Table 3). Moreover, higher percentage of spindles abnormalities (35.74%) was found in autotetraploid rice hybrid harboring the interaction at *Sb* pollen sterility locus than other hybrids with interactions at *Sa* and *Sc* pollen sterility loci. Straggling chromosomes and bridges were the primary chromosomal aberrations, and total abnormality percentage in autotetraploid rice hybrid caused by the interaction of *Sb* pollen sterility loci was more than 35% at Anaphase II (Table 3). Higher percentage of straggling chromosomes (35.74%) was found in autotetraploid rice hybrid harboring the interaction at *Sb* pollen sterility locus than other hybrids having interactions at *Sa* and *Sc* pollen sterility loci. In Telophase II, chromosome lagging, asynchronous meiocytes and micronucleus were the primary types of abnormalities, and abnormal cells were 2.87% and 13.79% in diploid and autotetraploid rice hybrids harboring interaction at *Sb* locus, respectively (Table 3). These results suggested that stronger pollen sterility effect existed in autotetraploid rice hybrids harboring interaction at *Sb* pollen sterility locus compared to diploid rice hybrid and other hybrids having interaction at single pollen sterility locus.

3.3. Gene expression profile changes in autotetraploid rice hybrids harboring allelic interaction at Sb pollen sterility locus

Cytological results indicated that higher percentage of abnormalities existed in autotetraploid rice hybrids harboring interaction at *Sb* pollen sterility locus, therefore, we next performed transcriptome profiling using Affymetrix Gene Chips to investigate pollen sterility effect. Here, three comparison groups, named IPE_{Sa} (*Sa*-4x vs *Sa*-2x), IPE_{Sb} (*Sb*-4x vs *Sb*-2x) and IPE_{Sc} (*Sc*-4x vs *Sc*-2x), were used to evaluate the interaction effects of *Sb* pollen sterility locus in autotetraploid and diploid rice hybrids (Fig. 3; Supplementary Table S2).

We identified the differentially expressed genes (2-fold at P value < 0.05) in autotetraploid rice hybrid compared with diploid rice hybrid. Among these groups, high numbers of differentially expressed genes (DEGs) were detected in IPE_{Sb}, and most of the genes displayed up-regulation in all groups (Fig. 3A). In the group of IPE_{Sa}, 126 and 76 genes were found to be up- and down-regulated, respectively (Fig. 3A). In the group of IPE_{Sc}, 190 and 33 genes were found to be up- and down-regulated, respectively (Fig. 3A). Interestingly, the largest numbers of

Table 2
Frequency of abnormal chromosome behaviors in diploid and autotetraploid rice hybrids at Metaphase I and Anaphase I.

Material	Interaction locus	Metaphase I			Anaphase I			
		No (n)	Normal (%)	Lagging (%)	No (n)	Normal (%)	Straggling (%)	Bridge (%)
E1-2x × E5-2x	*Sa*	307	98.27	1.73	259	97.57	0.33	2.11
E1-4x × E5-4x	*Sa*	220	97.76	2.24	267	96.93	2.51	0.56
E1-2x × E2-2x	*Sb*	271	99.79	0.21	201	98.01	0	1.99
E1-4x × E2-4x	*Sb*	154	97.40	2.60	123	96.75	2.44	0.81
E1-2x × E4-2x	*Sc*	258	98.32	1.68	269	97.89	0.85	1.26
E1-4x × E4-4x	*Sc*	327	97.34	2.66	342	96.49	1.87	1.64

Note: E1 represents Taichung 65, while E2, E4 and E5 represent near-isogenic lines of Taichung 65; 2x and 4x represent diploid and autotetraploid rice.

Table 3
Frequency of abnormal chromosome behaviors in diploid and autotetraploid rice hybrid at Metaphase II, Anaphase II and Telophase II.

Material	Interaction locus	Metaphase II					Anaphase II					Telophase II				
		No.	Normal (%)	Lagging (%)	Spindle (%)	Asynchronous Meiocytes (%)	No.	Normal (%)	Spindle (%)	Straggling (%)	No.	Normal (%)	Micronucleus (%)	Lagging (%)	Asynchronous Meiocytes (%)
E1-2x × E5-2x	*Sa*	260	94.36	2.14	2.86	0.64	191	93.54	1.78	4.60	285	98.26	0.51	1.23	0
E1-4x × E5-4x	*Sa*	291	77.35	2.36	20.18	0.23	325	88.12	9.56	2.32	312	89.24	0.12	4.63	6.01
E1-2x × E2-2x	*Sb*	318	90.88	0.63	8.18	0.31	196	93.37	1.02	5.61	383	97.13	1.83	1.04	0
E1-4x × E2-4x	*Sb*	235	62.98	1.28	35.74	0	191	67.02	8.38	24.61	174	86.21	0	9.77	4.02
E1-2x × E4-2x	*Sc*	259	93.56	1.24	4.97	0.23	276	94.65	2.15	3.20	213	97.56	0.32	2.12	0
E1-4x × E4-4x	*Sc*	325	76.33	5.64	17.53	0.50	296	77.23	3.23	19.54	354	87.06	1.56	8.12	3.26

Note: E1 represents Taichung 65, while E2, E4 and E5 represent near-isogenic lines of Taichung 65; 2x and 4x represent diploid and autotetraploid rice.

differentially expressed genes, including 446 and 144 up- and down-regulated genes were detected in IPE_{Sb} group, respectively (Fig. 3A).

GO enrichment analysis was further used to annotate the DEGs that were differentially expressed in diploid and autotetraploid rice hybrids having allelic interactions at three differential pollen sterility loci. In biological process, six prominent functional gene classes, such as development process related genes, response to stimulus and stress related genes, reproduction related genes, and biological regulation related genes, showed obvious differences among the interactions of different pollen sterility loci (Fig. 4). In cellular component, three prominent functional gene classes, namely cell, cell pert and organelle, exhibited significant differences among three single pollen sterility loci (Fig. 4). In molecular function, seven prominent functional gene classes, such as binding related genes, transcription regulator activity, transporter activity and catalytic activity genes, displayed significant differences among different pollen sterility loci (Fig. 4). These results suggested that *Sb* pollen sterility locus could lead to the higher number of DEGs than other two single pollen sterility loci. Transcription regulation activity, reproduction related functional gene classes and response related genes were significantly enriched in *Sb* pollen sterility locus compared to other two loci.

To validate the gene expression profiles data in diploid and autotetraploid rice, we selected nine differentially expressed genes using the qRT-PCR analysis. Based on the qRT-PCR analysis, the expression patterns of nine genes, including the five down-regulated genes (*Loc_Os04g40290*, *Loc_Os03g58600*, *Loc_Os05g37350*, *Loc_Os03g50520*, and *Loc_Os02g40440*) and four up-regulated genes (*Loc_Os03g11600, Loc_Os01g66890, Loc_Os07g06620,* and *Loc_Os04g51430*), which were differentially expressed in the autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus, were consistent with the expression levels detected in the transcriptome analysis. These results indicated that the expression levels of nine genes generated by qRT-PCR were consistent with transcriptome analysis, which demonstrated the reliability and accuracy of microarray results (Supplementary Fig. S2).

3.4. Differentially expressed genes specific to the interaction at Sb pollen sterility locus in autotetraploid compared to diploid rice hybrid

Transcriptome analysis was used to analyze the pollen sterility effect in autotetraploid rice hybrid compared to its corresponding diploid rice hybrid. In this study, we found that autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus had the largest number of DEGs compared with other two hybrids with interaction at *Sa* or *Sc* pollen sterility locus. To make sure that *Sb* pollen sterility locus (IPE_{Sb}) has the stronger pollen sterility effect in autotetraploid rice hybrid, we compared our results with the other two single pollen sterility locus (*Sa* and *Sc*). Therefore, we focused on the 446 DEGs that were specifically expressed in autotetraploid rice hybrid harboring *Sb* pollen sterility locus interaction (Fig. 3B; Supplementary Table S3). Predicated protein-protein interaction analysis was further used to evaluate the specific DEGs caused by the interaction at *Sb* pollen sterility locus. Based on the String database, 210 of these genes were predicated to undergo protein-protein interaction analysis and showed a strong interaction network (Supplementary Fig. S3). All of these results suggested that *Sb* pollen sterility locus had significant interaction effect on the pollen fertility of autotetraploid rice.

3.5. Transcription regulation related genes in autotetraploid rice hybrid harboring interaction at Sb pollen sterility locus compared to diploid hybrids

Transcription regulator activity was the significant GO category under the interaction of *Sb* pollen sterility locus; therefore, we focused on these transcription regulations related genes. In total, 30 genes were identified as transcription factors (TFs) and these genes mainly displayed up-regulation (Supplementary Table S4). Transcription factor related genes mainly divided into five major groups (Fig. 5). The first

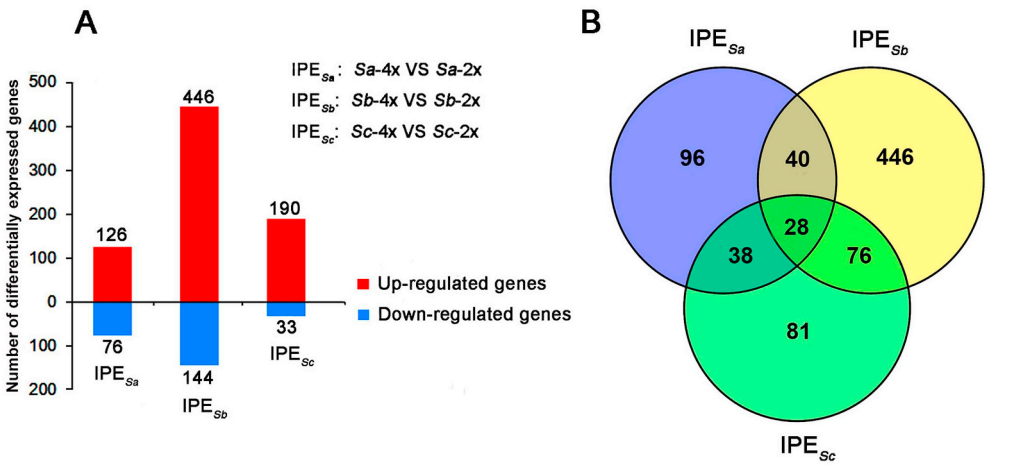


Fig. 3. Differentially expressed genes in autotetraploid rice hybrids detected by the different pollen sterility loci interaction compared to their diploid hybrids. (A) Number of differentially expressed genes in autotetraploid rice hybrids harboring interactions at three single pollen sterility loci. Genes were divided into up- and down-regulated if their expression levels were increased and decreased two fold at P-value < 0.05, respectively. (B) Venn diagram of differentially expressed genes in autotetraploid rice hybrids harboring interaction of different pollen sterility locus.

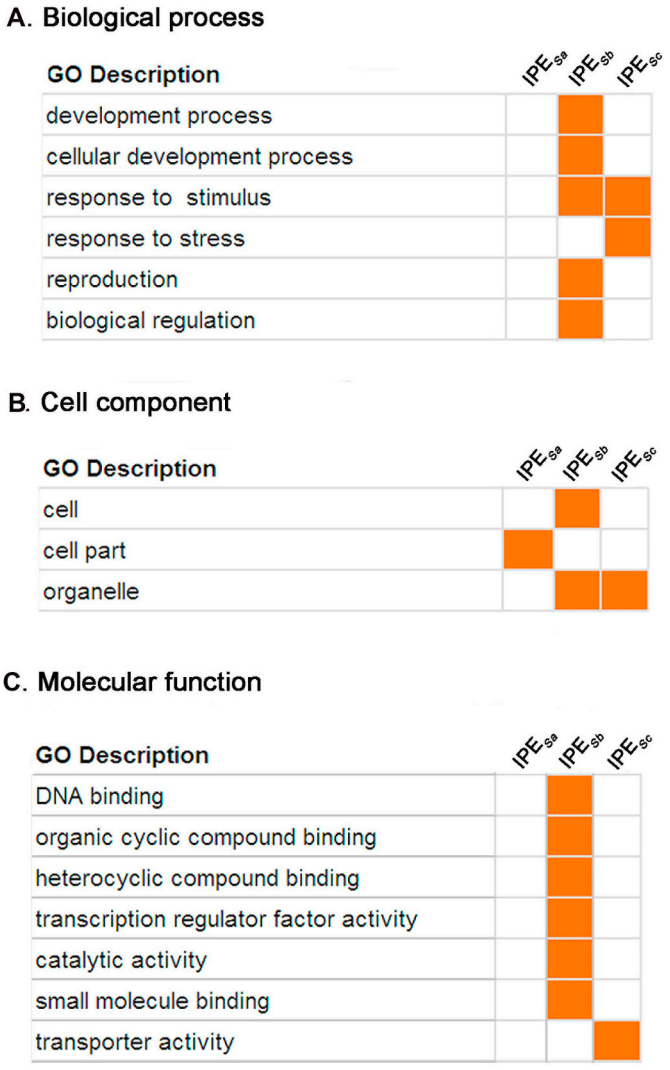


Fig. 4. GO analysis of differentially expressed genes in autotetraploid rice hybrid harboring interaction of three single pollen sterility loci compared to their diploid hybrids. Genes were divided into three categories: biological process, cellular component, and molecular function.

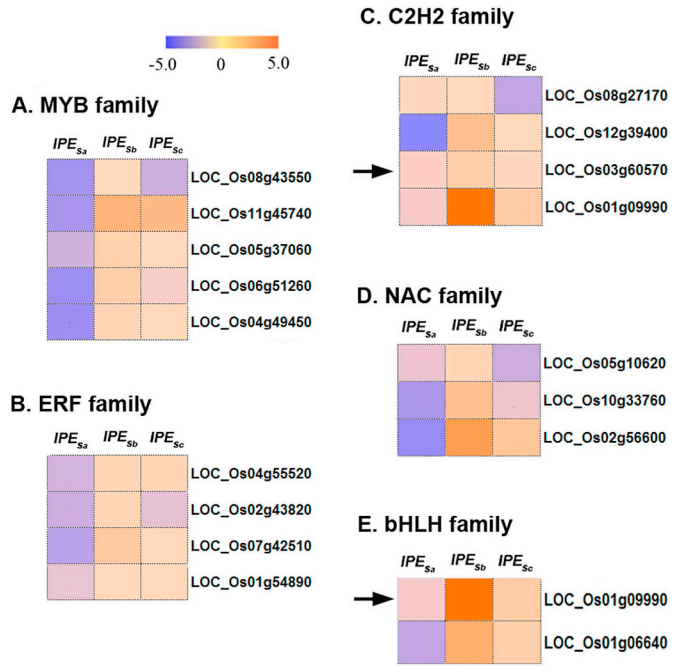


Fig. 5. Expression patterns of genes involved in transcription regulation in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus. (A) Expression patterns of MYB family genes. (B) Expression patterns of ERF family genes. (C) Expression patterns of C2H2 family genes. (D) Expression patterns of NAC family genes. (E) Expression patterns of bHLH family genes.

group was comprised of five MYB family genes, and all of these genes (*LOC_Os08g43550*, *LOC_Os11g45740*, *LOC_Os05g37060*, *LOC_Os06g51260*, and *LOC_Os04g49450*) showed up-regulation (Fig. 5A). The second group contained five AP2/ERF superfamily (ERF) genes, and four of these genes, namely *LOC_Os04g55520*, *LOC_Os02g43820*, *LOC_Os07g42510*, and *LOC_Os01g54890*, displayed up-regulation (Fig. 5B). Four C2H2 zinc-finger domain protein family (C2H2) genes (*LOC_Os08g27170*, *LOC_Os12g39400*, *LOC_Os03g60570* and *LOC_Os01g09990*) were detected in group three and all of them showed up-regulation (Fig. 5C). The fourth group was comprised of three NAM, ATAF, and CUC (NAC) transcription factor genes, and all of these genes (*LOC_Os05g10620*, *LOC_Os10g33760*, and *LOC_Os02g56600*) showed up-regulation (Fig. 5D). The fifth group composed of two genes (*LOC_Os01g09990* and *LOC_Os01g06640*) encoding the basic/helix-loop-helix proteins (bHLH), which also exhibited up regulation (Fig. 5E).

We further annotated these transcription regulations related genes, and found that two genes (*LOC_Os01g09990* and *LOC_Os03g60570*) were functionally analyzed and involved in the pollen development and pollen fertility (Fig. 5). For example, *ZFP15* (*LOC_Os03g60570*) encodes a C2H2 zinc finger protein, and play important role in the pollen development (Huang et al., 2005). *OsbHLH025* (*LOC_Os01g09990*)

encodes a helix-loop-helix DNA-binding domain containing protein, and over-expression of this gene could cause low fertility in rice (Yamamura et al., 2015).

3.6. Meiosis-specific differentially expressed genes in autotetraploid rice hybrid harboring interaction at Sb pollen sterility locus compared to diploid hybrid

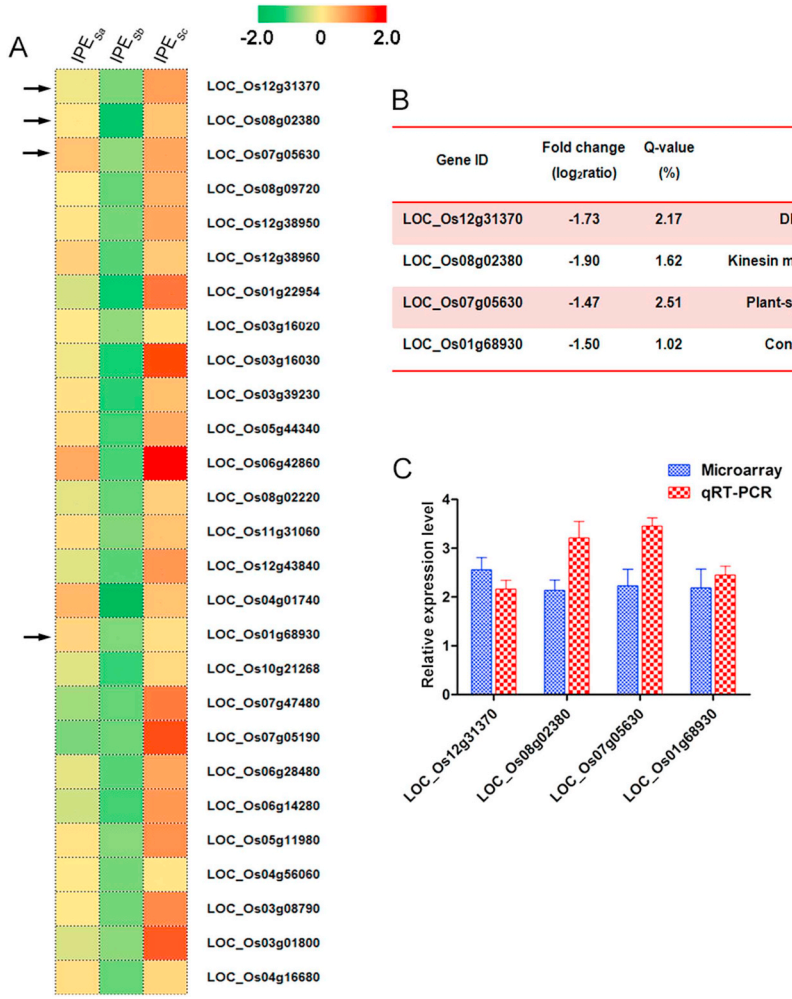
As strong pollen sterility effect produced by *Sb* pollen sterility locus in autotetraploid rice hybrid, we combined specific DEGs generated by the interaction at *Sb* pollen sterility locus with rice anther meiosis stage-specific genes and meiosis-related genes, which were verified by high throughput gene expression data (Aya et al., 2011; Fujita et al., 2010; Deveshwar et al., 2011; Yant et al., 2013; Wu et al., 2014, 2015; Wright et al., 2015; Guo et al., 2017; Li et al., 2017). In this study, we identified 139 meiosis stage-specific genes and meiosis-related genes, and these genes displayed at least two fold changes in autotetraploid rice hybrid compared with diploid rice hybrid (Supplementary Table S5). Among these 139 genes, several genes encoded the meiosis-related proteins and it mainly involved in the pollen development and pollen fertility. Additionally, a strong interaction network was also detected among meiosis related genes (Supplementary Fig. S4).

Moreover, 27 DEGs displayed down-regulation, including several genes which had been demonstrated as associated with low pollen fertility in the previous studies (Fig. 6A). For example, *RAD51A2* (*LOC_Os12g31370*) is a meiosis-specific DNA pairing and repair related gene, and it encodes a DNA repair protein (*Rad51*) during the meiosis (Fig. 6B and C). *PSS1* (*LOC_Os08g02380*) encodes a kinesin motor domain containing protein and play important role in meiosis (Fig. 6B and C). *MIL1* (*LOC_Os07g05630*) encodes an OsGrx_C10-glutaredoxin subgroup III expressed protein and play important role in meiosis (Fig. 6B and C). *OsXRI1* (*LOC_Os01g68930*) encodes a conserved hypothetical protein, and crucial for the meiosis process in *Arabidopsis thaliana* (Fig. 6B and C). All of these results suggested that pollen sterility caused by the interaction at *Sb* pollen sterility locus play important role and might be the major reason for pollen abortion in autotetraploid rice hybrids.

4. Discussion

4.1. Interaction at Sb pollen sterility locus is stronger in autotetraploid than diploid rice hybrid

Inter-subspecific autotetraploid rice hybrids have stronger yield potential and greater adaptability, compared with the diploid rice hybrid (Tu et al., 2007; Shahid et al., 2011, 2012; Wu et al., 2013). Allelic interaction between *i* (*indica*) and *j* (*japonica*) alleles at *Sa*, *Sb* and *Sc* pollen sterility loci, were thought to be primary cause of the low pollen



B

Gene ID	Fold change (log_2ratio)	Q-value (%)	Annotation	Expression level
LOC_Os12g31370	-1.73	2.17	DNA repair protein Rad51	Down
LOC_Os08g02380	-1.90	1.62	Kinesin motor domain containing protein	Down
LOC_Os07g05630	-1.47	2.51	Plant-specific CC-type glutaredoxin	Down
LOC_Os01g68930	-1.50	1.02	Conserve hypothetical protein	Down

Fig. 6. Expression patterns of genes associated with meiosis process in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus. (A) Expression patterns of putative meiosis stage and meiosis specific genes. Genes indicated by arrows are meiosis related genes, while other genes are meiosis stage specific. (B) Annotation and expression levels of primary DEGs related to meiosis identified from autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus. (C) qRT-PCR confirmation of meiosis-related genes in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus.

fertility in autotetraploid rice hybrids (He et al., 2011b; Wu et al., 2015). Pollen fertility of diploid rice hybrid, harboring interaction at *Sb* pollen sterility locus, was much lower than other pollen sterility locus (i.e., *Sa* or *Sc*) (Zhang et al., 2006; Shahid et al., 2013b). In the present work, we developed the autotetraploid and diploid rice hybrids using the near-isogenic lines which were heterozygous at *Sb* pollen sterility locus to evaluate the pollen sterility effect.

Here, we detected the pollen fertility of diploid and autotetraploid rice hybrids harboring interaction at *Sb* pollen sterility locus. Our results indicated that pollen fertility of autotetraploid rice hybrid caused by interaction at *Sb* pollen sterility locus was only 30.10%, which was much lower than its parents. Moreover, we observed significant differences between autotetraploid and diploid rice hybrid at *Sb* pollen sterility locus compared to *Sa* and *Sc* loci. This result was consistent with the previous study, which indicated that pollen fertility of autotetraploid rice hybrid caused by interactions at *Sb* pollen sterility locus was much lower than other single pollen sterility locus (i.e. *Sa* and *Sc*) (Zhao et al., 2006). In diploid rice hybrid, allelic interaction of *i* (*indica*) and *j* (*japonica*) alleles at *Sb* locus mainly lead to the abortive pollens at middle microspore stage (Zhang et al., 2006). Here, early stage abortion and higher percentage of abnormalities were observed in autotetraploid rice hybrid than diploid counterpart harboring interaction at *Sb* pollen sterility locus using the WE-CLSM observation. Cell degeneration, cell shrinkage, irregular-shaped cells, and callose without disassembly were frequently observed at Metaphase II in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus. These results indicated that interaction at *Sb* pollen sterility locus in autotetraploid rice probably have a higher pollen sterility effect than in diploid rice hybrid.

Chromosome behavior analysis during meiosis stage was also observed to verify the interaction effects of *Sb* pollen sterility locus compared to other single pollen sterility locus (*Sa* and *Sc*) in autotetraploid and its diploid rice hybrid. We observed the abnormal male meiocytes during the five meiosis stages, including the Metaphase I, Anaphase I, Metaphase II, Anaphase II and Telophase II. The chromosome lagging in Metaphase I, chromosome straggling and spindle abnormality in Anaphase I, chromosome lagging, abnormal spindles, and asynchronous meiocytes in Metaphase II, spindle abnormalities and chromosome straggling in Anaphase II, chromosomes lagging and asynchronous meiocytes in Telophase II were observed, and these were the primary chromosomal abnormalities among the five meiotic stages. Cytological results indicated that percentage of abnormal cells in autotetraploid rice hybrid under the interaction of *Sb* pollen sterility was higher than the diploid rice hybrid. For example, the frequencies of abnormal cells were about 37.02% and 32.28% in autotetraploid rice hybrid while only 9.12% and 6.63% were observed in diploid rice hybrid at Metaphase II and Anaphase II, respectively. Additionally, chromosome behavior observation in Telophase II demonstrated that lower percentage of abnormalities in autotetraploid rice hybrid and this might be due to the short duration of this stage because chromosomes have already moved towards the opposite poles.

We also observed the abnormal male meiocytes in autotetraploid rice and its diploid hybrid harboring interactions at *Sa* and *Sc* pollen sterility loci in the present study. Cytological results indicated that percentage of abnormal cells during Metaphase II and Anaphase II in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus was much higher than *Sa* and *Sc* pollen sterility locus. These results was consistent with the WE-CLSM results and showed that Meiosis II was the pollen abortion stage and higher percentage of abnormalities were observed in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus (Wu et al., 2017). All of these results demonstrated that allelic interaction of *i* (*indica*) and *j* (*japonica*) alleles at *Sb* locus exhibited higher pollen sterility in autotetraploid rice hybrid than its diploid rice hybrid and other hybrids having interaction at single pollen sterility locus.

4.2. Differential molecular mechanism is probably existed in autotetraploid rice hybrids harboring interaction at Sb pollen sterility locus

Pollen sterility is a major hindrance in the utilization of autotetraploid rice hybrid (He et al., 2011b). *Sb* locus was thought to be one of important pollen sterility locus that leads to higher pollen abortion than other pollen sterility loci in autotetraploid rice hybrid (Zhao et al., 2006). However, the molecular mechanism of genetic interaction at *Sb* pollen sterility locus in autotetraploid rice hybrid is not fully known. We employed the transcriptome analysis to evaluate the genome wide alterations and their relationship with pollen sterility in autotetraploid rice hybrid and its diploid rice. Using the bright-field microscopy with 4',6-diamidino-2-phenylindole (DAPI) fluorescence staining, and laser capture of individual cells have made it possible to dissect pollen mother cells (Fujita et al., 2010; Tang et al., 2010; Yang et al., 2011; Wu et al., 2014, 2015). In this study, we used the DAPI fluorescence staining and WE-CLSM techniques to detect the precise meiotic stages for transcriptome analysis, and then analyzed the pollen sterility effect caused by genetic interactions at *Sb* pollen sterility locus in autotetraploid and its diploid rice hybrid.

Among the differentially expressed genes (DEGs), 590 genes were detected in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus, and the DEGs were much higher than other pollen sterility locus. These specific DEGs were further used to evaluate the pollen sterility effects in autotetraploid rice hybrid caused by different loci. Notably, 446 DEGs were specifically expressed in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus compared with other two pollen sterility loci. We used the predicted protein-protein interaction analysis to evaluate the pollen sterility effect at *Sb* pollen sterility locus, and found that 210 DEGs were involved in the strong interaction-interaction network. These results displayed that interaction effects caused by *Sb* pollen sterility locus was higher than other pollen sterility loci.

GO analysis was further used to annotate the DEGs associated with the interactions at *Sa*, *Sb* and *Sc* pollen sterility loci. Compared to other two pollen sterility loci, significant variations were detected at *Sb* pollen sterility locus. Three GO categories, including the transcription regulation activity, reproduction related functional gene classes and response related genes were mainly involved in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus. The previous studies have shown that transcription regulation activity and reproduction related functional gene classes were involved in the fertility regulation of autotetraploid rice (Wu et al., 2015, 2017). Transcription factor activity was one of the significant categories in autotetraploid rice hybrids compared to other single pollen sterility locus. BHLH and MYB related families were the primary gene classes detected in our study and involved in the pollen development and pollen fertility. For example, *OsbHLH025* (*LOC_Os01g09990*), encodes a helix-loop-helix DNA-binding domain containing protein, and cause low fertility in rice (Yamamura et al., 2015). *ZFP15* (*LOC_Os03g60570*) encodes a C2H2 zinc finger protein and paly important role in rice pollen development (Huang et al., 2005).

As for the higher pollen sterility effect exhibited at *Sb* pollen sterility locus mainly began at early meiotic stage, we compared our DEGs with the meiosis related and meiosis stage specific genes detected in the previous studies (Fujita et al., 2010; Deveshwar et al., 2011; Hollister et al., 2012; Yant et al., 2013; Wu et al., 2014, 2015; Wright et al., 2015). In the present work, 139 DEGs were involved in the meiosis process in autotetraploid rice hybrid compared to diploid rice hybrid. We further annotated these genes and found several important genes that encoded the meiosis-related proteins and mainly involved in the pollen development and pollen fertility. Moreover, one strong co-expression network was also found in these meiosis-related genes. These results clearly demonstrated that interaction at *Sb* pollen sterility locus in autotetraploid rice hybrid could result in higher number of differentially expressed genes than other two single pollen sterility locus.

These results displayed remarkable differences between the interaction effects caused by *Sb* pollen sterility locus and other pollen sterility loci.

4.3. Down-regulation of meiosis-related genes were probably the primary reason for the strong interaction at Sb pollen sterility locus in autotetraploid rice hybrid

Meiosis plays an important role in rice pollen development. To detect the expression pattern of meiosis-related genes is thought to be an effect way to reveal the cause of low pollen fertility in autotetraploid rice hybrid (Wu et al., 2014; Guo et al., 2017). In our previous study, 335 genes displayed differential expressions in meiosis stage compared with the pre-meiotic interphase and microspore stage during rice pollen development in autotetraploid rice (Wu et al., 2014). Moreover, 42 meiosis-related genes exhibited polyploidy specific expression patterns in neo-tetraploid rice (Guo et al., 2017). Here, *Sb* pollen sterility locus exhibited a strong pollen sterility effect in autotetraploid rice and 139 DEGs were involved in meiosis compared to its diploid rice hybrid.

Here, our main focus was on the 139 meiosis-related or stage-specific DEGs associated with the *Sb* pollen sterility locus. We compared our results with the previous studies in autotetraploid rice (Wu et al., 2014; Guo et al., 2017), and 105 genes were detected in autotetraploid rice during the meiosis process. For example, 93 meiosis-related and stage-specific genes showed same expression tendency as Wu et al. (2014), and 22 meiosis-related and stage-specific genes showed similar tendency to Guo et al. (2017). Notably, 27 genes have been detected that were involved in meiosis. For example, *RAD51A2* (*LOC_Os12g31370*) encodes a meiosis-specific DNA pairing and repair protein (*Rad51*) during the meiosis (Xu et al., 2018). *PSS1* (*LOC_Os08g02380*) encodes a kinesin motor domain containing protein and play an important role in meiosis (Zhou et al., 2011). *MIL1* (*LOC_Os07g05630*) encodes an OsGrx_C10-glutaredoxin subgroupIII expressed protein and involved in meiosis process (Hong et al., 2012). *OsXRI1* (*LOC_Os01g68930*) encodes a conserved hypothetical protein, and crucial for the meiosis process in *Arabidopsis thaliana* (Jin et al., 2013). These results suggested that *Sb* pollen sterility locus play a critical role in pollen abortion, and down-regulation of meiosis-related genes might be the major reason for pollen abortion in autotetraploid rice hybrid harboring interaction at *Sb* pollen sterility locus.

5. Conclusions

In the present study, we found that interaction at *Sb* pollen sterility locus exhibited higher pollen sterility in autotetraploid rice hybrid compared with its diploid rice hybrid and other single pollen sterility locus. Our results provided strong evidence for the high pollen sterility effect of *Sb* pollen sterility locus by using the cytological and transcriptome analyses in autotetraploid rice hybrids. Differentially expressed genes, including 27 meiosis related genes, can be used as candidate genes to reveal the mechanism of *Sb* pollen sterility locus in autotetraploid rice hybrids.

Author Contributions

Conceived and designed the experiments: XDL. Performed the experiments: JWW, MQS, MYC, XL, JRL, XSX and SSD. Analyzed the data: JWW, MQS and XDL. Contributed reagents/materials/analysis tools: XDL. Wrote the paper: JWW, MQS and XDL.

Disclosure statement

The authors have declared that no competing interests exist.

Acknowledgments

This work was supported by the NSFC to XD Liu (31571625), the Guangzhou Science and Technology Key Program to XD Liu (201707020015), the Guangdong Science and Technology Program to XD Liu (2017A030303069), and the Guangdong Science and Technology Program to JW Wu (2018A030313644). The authors were grateful to Professor Guiquan Zhang for providing Taichung65–2x and its iso-genic lines, E2, E4 and E5. Ms. Shuhong Yu developed and cultivated Taichung65–4x and its autotetraploid rice iso-genic lines, E2-4x, E4-4x and E5-4x. We also thank Dr. Zhixiong Chen, Dr. Lan Wang and other lab members for assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.019.

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