

RESEARCH PAPER

# Allelic interaction of F<sub>1</sub> pollen sterility loci and abnormal chromosome behaviour caused pollen sterility in intersubspecific autotetraploid rice hybrids

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## Abstract

The intersubspecific hybrids of autotetraploid rice has many features that increase rice yield, but lower seed set is a major hindrance in its utilization. Pollen sterility is one of the most important factors which cause intersubspecific hybrid sterility. The hybrids with greater variation in seed set were used to study how the F<sub>1</sub> pollen sterile loci (*S-a*, *S-b*, and *S-c*) interact with each other and how abnormal chromosome behaviour and allelic interaction of F<sub>1</sub> sterility loci affect pollen fertility and seed set of intersubspecific autotetraploid rice hybrids. The results showed that interaction between pollen sterility loci have significant effects on the pollen fertility of autotetraploid hybrids, and pollen fertility further decreased with an increase in the allelic interaction of F<sub>1</sub> pollen sterility loci. Abnormal ultra-structure and microtubule distribution patterns during pollen mother cell (PMC) meiosis were found in the hybrids with low pollen fertility in interphase and leptotene, suggesting that the effect-time of pollen sterility loci interaction was very early. There were highly significant differences in the number of quadrivalents and bivalents, and in chromosome configuration among all the hybrids, and quadrivalents decreased with an increase in the seed set of autotetraploid hybrids. Many different kinds of chromosomal abnormalities, such as chromosome straggling, chromosome lagging, asynchrony of chromosome disjunction, and tri-fission were found during the various developmental stages of PMC meiosis. All these abnormalities were significantly higher in sterile hybrids than in fertile hybrids, suggesting that pollen sterility gene interactions tend to increase the chromosomal abnormalities which cause the partial abortion of male gametes and leads to the decline in the seed set of the autotetraploid rice hybrids.

**Key words:** Autotetraploid rice, chromosome behaviour, hybrid sterility, microtubule, *Oryza sativa*, pollen sterility, ultra-structure.

## Introduction

Polyploidy is a potent source of increasing crop productivity and plays a significant role in plant breeding programmes. Over 70% of all angiosperm species have an increase in ploidy level somewhere in their evolutionary histories. Some polyploids are of recent origin, while others are millions of years old (Levin, 2002). Polyploidy provides genome buffering, increased allelic diversity, permits novel phenotypic variation to be generated, and is often accompanied by a loss of duplicated chromatin and changes in gene expression (Udall

and Wendel, 2006). Polyploidy has abundant advantages over diploid crops such as an increase in greenness, lushness, seed index, and biomass yield (Bingham *et al.*, 1994).

Autotetraploid rice has great potential to increase the yield of rice and has more nutrition and is more resistant to insect pests and diseases than diploid rice (Chen *et al.*, 1987; Song and Zhang, 1992). Autotetraploid rice depicted greater genetic variation than diploid rice (Luan *et al.*, 2008) and greater grain length and width than diploid rice (Tu *et al.*, 2007; Shahid

*et al.*, 2011). Intersubspecific autotetraploid rice hybrids have more hybrid vigour and are more stable across different environments than diploid hybrids (Shahid *et al.*, 2011). However, lower seed set is the major hindrance in its utilization. Pollen fertility has a significant correlation with seed set and autotetraploid rice has lower pollen fertility than diploid rice (Shahid *et al.*, 2010). Therefore, it is very important to find the reasons for lower pollen fertility in autotetraploid rice.

Asian cultivated rice could be divided into three subspecies, i.e. *indica*, *japonica*, and *javanica* (Chang, 1976). Intersubspecific crosses of Asian cultivated rice exhibits one of the best hybrid combinations in the rice crop, however, the utilization of their hybrid vigour is limited due to the presence of partial to complete sterility (Kato *et al.*, 1928; Zhang *et al.*, 1997; Xiao and Yuan, 2009). Cytological studies revealed that the male gamete abortions (Zhang *et al.*, 1993; Liu *et al.*, 2004a), female gamete abortions (Liu *et al.*, 2004a), and reduced dehiscence of the anthers (Maekawa *et al.*, 1997; Zhang *et al.*, 2006) could cause the hybrid sterility. Pollen sterility is one of the most important reasons of intersubspecific hybrid sterility and contributed equally to the low fertility of intersubspecific hybrids compared with embryo sac sterility (Yan *et al.*, 2003, Song *et al.*, 2005). At least six loci, i.e. *S-a*, *S-b*, *S-c*, *S-d*, *S-e*, and *S-f* have been mapped for pollen sterility in intersubspecific hybrids (Zhang *et al.*, 1993, 1994). The *S-a* gene has been cloned (Long *et al.*, 2008) and the *S-b* (Li *et al.*, 2006) and *S-c* (Yang *et al.*, 2004) genes have been fine mapped. These gene interactions only related to pollen sterility and had no relation with embryo sac sterility and their cytological mechanism is different at different loci (Zhang *et al.*, 2006). Pollen abortion in intersubspecific hybrids was due to allelic interaction of the F<sub>1</sub> pollen sterility locus, and the F<sub>1</sub> pollen sterility alleles was in accordance with a one-locus sporogametophytic interaction model (Zhang and Lu, 1993). Typical *indica* and *japonica* rice carry *S<sup>i</sup>* and *S<sup>j</sup>* alleles at F<sub>1</sub> pollen sterility loci, respectively. The gametes with genotypes *S<sup>i</sup>S<sup>i</sup>* and *S<sup>j</sup>S<sup>j</sup>* in the hybrids are fertile and the gametes having the *S<sup>j</sup>* allele in the hybrid genotype *S<sup>i</sup>S<sup>j</sup>* are partially aborted (Oka, 1974; Sano, 1990; Zhang *et al.*, 2006). The alleles that show good affinity with *indica* and *japonica* varieties and do not interact with alleles *S<sup>j</sup>* (*japonica*) and *S<sup>i</sup>* (*indica*) are called neutral alleles (*S<sup>n</sup>*), i.e. *S<sup>n</sup>* is compatible with both *S<sup>i</sup>* and *S<sup>j</sup>* and no allelic interaction happens between *S<sup>n</sup>* and *S<sup>j</sup>* or *S<sup>i</sup>* (Ding *et al.*, 2003; Shi *et al.*, 2009). The neutral alleles *S<sup>n-a</sup>* (Long, 2007) and *S<sup>n-b</sup>* (Shi *et al.*, 2009) have been found in cultivated rice (Guanglu'ai 4) and a wild rice accession (GZW099) of *Oryza rufipogon* Griff. indigenous to Gaozhou, Guangdong, respectively.

Pollen grains play a vital role in plant fertility through the generation and delivery of the male gametes to the embryo sac for double fertilization in flowering plants (Borg *et al.*, 2009). Chromosome behaviour during meiosis played an important role in normal male gamete formation in the mutants of diploid plants (Kunio and Takeshi,

1983). Plant fertility in the induced autotetraploid of kale depends on the chromosome pairing at meiosis (Jenczewski *et al.*, 2002). Chromosome behaviour played a very important role in fertility at the cell level (Srivastava, 1956; Alley, 1957; Wolf *et al.*, 1989; Lavania *et al.*, 1991; Luo *et al.*, 1992; Srivastava *et al.*, 1992). Bremer and Bremer-Reinders (1954) reported that an increase in the fertility of autotetraploid *Secale cereale* was correlated with an increase in bivalents and a decrease in univalents and multivalents during meiosis. Quadrivalents and bivalents have significant importance in the fertility of polyploids (Jones, 1994). Venkateswarlu and Rao (1976) made a comparative study between the colchicine-induced 'raw' autotetraploid *Coix lacryma* (S<sub>0</sub> generation) and the S<sub>3</sub> generation (evolved from S<sub>0</sub> through selection for vigour and fertility after the selfing-cross of three successive generations). They found a significant decrease in the frequency of quadrivalents and an increase in ring quadrivalents and further suggested that selection for vigour and fertility could increase the number of ring quadrivalents in autotetraploids and that ring quadrivalents are likely to have a more regular disjunction at anaphase I. Our previous studies also showed that abnormal chromosome behaviour resulted in low pollen fertility of intersubspecific autotetraploid rice hybrids (He *et al.*, 2011).

Our previous research revealed that the interaction of *S-b* might also affect the pollen fertility of intersubspecific autotetraploid rice hybrids (Zhao *et al.*, 2006). However, no detailed information was available about how single or polygene interactions affect the pollen fertility of autotetraploid rice hybrids. Moreover, little is known about the relationship between the interaction of sterile pollen genes and abnormal chromosome behaviour during pollen mother cell meiosis and how they affect the pollen fertility of autotetraploid rice. Some high seed set autotetraploid rice hybrids have been developed, so hybrids with significant variation in seed set were used for this research. The present study was conducted (i) to determine the genotypes of pollen sterility loci (*S-a*, *S-b*, and *S-c*) and their effect on pollen fertility; (ii) to determine the ultra-structural changes in pollen mother cells (PMC) and microtubule organization and chromosome behaviour during meiosis; and (iii) to find a relationship between chromosome behaviour and pollen sterility loci interaction and their effect on seed setting of autotetraploid rice hybrids.

## Materials and methods

### Plant materials

A total of four autotetraploid rice lines (Guanglu'ai 4-4x, E24-4x, L202-4x, and Jackson-4x) (Table 1) were used to conduct the study and five intersubspecific autotetraploid rice hybrids were developed from these lines (Table 2). Diploid rice varieties (Guanglu'ai 4; E24) and an autotetraploid rice (Guanglu'ai 4-4x) were used as controls. All materials were planted at the farm of the South China Agricultural University (SCAU). Row to row and plant to plant distances were kept as 20 cm and 17 cm, respectively. All the field practices were done according to the recommendation of area.

**Table 1.** Parent's genotypes at *S-a*, *S-b*, and *S-c* loci

Name	<i>S-a</i> <sup>(a)</sup>	<i>S-b</i> <sup>(a)</sup>	<i>S-c</i> <sup>(a)</sup>	Subspecies
Guanglu'ai 4-4x	$S^n-aS^n-a S^n-aS^n-a$ <sup>(b)</sup>	$S^i-bS^i-b S^i-bS^i-b$	$S^i-cS^i-c S^i-cS^i-c$	<i>indica</i>
L202-4x	$S^i-aS^i-a S^i-aS^i-a$	$S^i-bS^i-b S^i-bS^i-b$	$S^i-cS^i-c S^i-cS^i-c$	<i>javanica</i>
Jackson-4x	$S^i-aS^i-a S^i-aS^i-a$	$S^i-bS^i-b S^i-bS^i-b$	$S^i-cS^i-c S^i-cS^i-c$	<i>javanica</i>
E24-4x	$S^i-aS^i-a S^i-aS^i-a$	$S^i-bS^i-b S^i-bS^i-b$	$S^i-cS^i-c S^i-cS^i-c$	<i>japonica</i>

<sup>a</sup>  $S^n$  indicates neutral allele;  $S^i$  indicates *indica* allele;  $S^j$  indicates *japonica* allele.

<sup>b</sup> Cited from the results of Long (2007).

**Table 2.** Hybrid's genotypes at *S-a*, *S-b*, and *S-c* loci and their pollen fertility and seed set

Hybrids	Type of cross	<i>S-a</i> <sup>(a)</sup>	<i>S-b</i> <sup>(a)</sup>	<i>S-c</i> <sup>(a)</sup>	No. of locus <sup>(b)</sup>	Pollen fertility (%)±SD	Seed set (%)±SD
Guanglu'ai 4-4x×L202-4x	<i>indica</i> × <i>javanica</i>	$S^n-aS^n-a S^i-aS^i-a$	$S^i-bS^i-bS^i-bS^i-b$	$S^i-cS^i-c S^i-cS^i-c$	1	96.0±0.3	73.1±6.4
Guanglu'ai 4-4x×Jackson-4x	<i>indica</i> × <i>javanica</i>	$S^n-aS^n-a S^i-aS^i-a$	$S^i-bS^i-bS^i-bS^i-b$	$S^i-cS^i-c S^i-cS^i-c$	1	94.7±3.3	70.1±4.0
Guanglu'ai 4-4x×E24-4x	<i>indica</i> × <i>japonica</i>	$S^n-aS^n-a S^i-aS^i-a$	$S^i-bS^i-bS^i-bS^i-b$	$S^i-cS^i-cS^i-cS^i-c$	2	67.4±8.3	58.6±1.8
L202-4x×E24-4x	<i>javanica</i> × <i>japonica</i>	$S^i-aS^i-aS^i-aS^i-a$	$S^i-bS^i-bS^i-bS^i-b$	$S^i-cS^i-cS^i-cS^i-c$	3	34.6±5.6	11.8±0.1
Jackson-4x×E24-4x	<i>javanica</i> × <i>japonica</i>	$S^i-aS^i-aS^i-aS^i-a$	$S^i-bS^i-bS^i-bS^i-b$	$S^i-cS^i-cS^i-cS^i-c$	3	12.9±4.4	24.6±6.4

<sup>a</sup>  $S^n$  indicates neutral allele;  $S^i$  indicates *indica* allele;  $S^j$  indicates *japonica* allele.

<sup>b</sup> Total number of heterozygous genotypes ( $S^i/S^i$ ) in a hybrid at the *S-a*, *S-b*, and *S-c* loci.

#### Detection of parents and hybrid genotypes at the *S-a*, *S-b* and *S-c*

**Marker selection:** Polymorphic pairs of primers were selected at the *S-a*, *S-b*, and *S-c* loci. The *S-a* gene has been cloned and the other two loci have been fine mapped, so closely linked markers to these genes were used. Two SNP markers (G02-76.3 and G02-78.5) were selected at the *S-a* locus (Long *et al.*, 2008), two markers (A07-55 and A07-130) at the *S-b* locus (Shi *et al.*, 2009; Wang, 2010), and four markers (P24-85.7, P24-100.7, P24-90.1, and P24-90.3) at the *S-c* locus (Wang, 2010).

**PCR amplification:** Young leaves were collected from autotetraploid rice and genomic DNA was extracted according to the SDS simple extraction method of Shi *et al.* (2009) with some minor modifications. The reaction reagent of 20 µl included 0.15 µmol l<sup>-1</sup> SSR primer, 0.2 mmol l<sup>-1</sup> dNTP, 1× PCR buffer (50 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> TRIS-HCl pH 8.3, 1.5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, and 0.01% glutin), 30–50 ng template DNA, and 1 U *Taq* polymerase. PCR amplification was performed with the PTC-100 PCR machine with the following profile: 5 min at 94 °C to denature; followed by 32 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and a final extension period of 72 °C for 5 min to complete the PCR reaction. PCR products were separated with 6% polyacrylamide gels and detected according to Li *et al.* (2002).

**Ultra-structural observation on PMC before and during meiosis:** Spikelets of 2–3 mm in length were collected and used to prepare the ultrathin sections according to the method of Liu *et al.* (1997). Ultrathin sections were observed under a TECNAI G2 12 Transmission Electronic Microscope (TEM).

#### Chromosome behaviour observation

**Fixation:** Inflorescences were collected from the shoots of rice plants with 1–4 cm between their flag leaf cushion and the second-to-last leaf cushion, and fixed in Carnoy solution (ethanol:acetic acid, 3:1 v/v) for at least 24 h. The samples were then stored in 70% ethanol at 4 °C.

**Chromosome staining:** Anthers were removed from the floret using forceps and a dissecting needle and placed in a small drop of 1 mg l<sup>-1</sup> 4', 6-diamidino-2-phenylindole (DAPI) on a glass slide. After 1 min, the glass slide was covered with a slide cover and was examined under a fluorescence microscope (Leica DMRXA). Meiosis stages were classified and explained according to He *et al.* (2011).

#### Microtubule observation

Microtubules were observed according to the method of Liu *et al.* (2004b) with some minor modifications and the details are as follows.

**Fixation:** Inflorescences were collected from rice shoots of plants with c. 1–4 cm between their flag leaf cushion and their second-to-last leaf cushion. Anthers were dissected out from the panicles, and were fixed in 4% paraformaldehyde in PEMS buffer [containing 10% DMSO, 400 µmol l<sup>-1</sup> *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), and 1% Triton X-100] for 1–2 h. After fixation, anthers were rinsed three times in PEMS solution (PEMS buffer+10% DMSO+1% Triton X-100), and were transferred to PEM solution (without DMSO, Triton, and MBS) and distilled water for washing (three times each). The anthers were then dehydrated in an alcohol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, and 100%).

**Infiltration and embedding:** After dehydration, the samples were infiltrated in a stepwise graded polyethylene glycol (PEG) mixture:alcohol solution (2:1, 1:1, 1:2 v/v) for 40 min each at 55 °C and then twice in pure PEG mixture for 1 h each at 55 °C. After that, samples were transferred in a tube for 1 h containing a pure PEG mixture. The PEG mixture contained PEG4000:PEG1500=2:3. Finally, the tubes were placed at room temperature until the materials hardened.

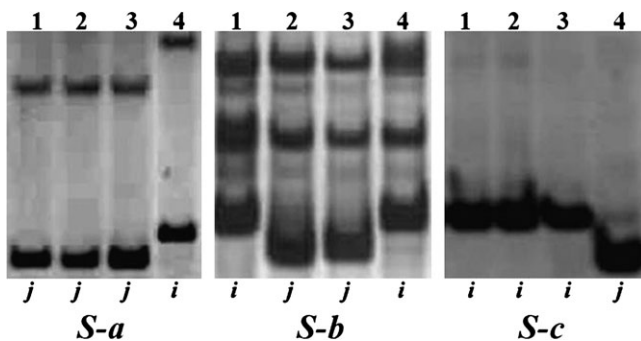
**Sectioning and indirect immunofluorescence labelling of microtubules:** After PEG embedding, sections were cut about 45–50 µm using a Leica CM 1850 freezing microtome. After rinsing 3–5 times in distilled water and three times in phosphate-buffered

saline (PBS) solution, sections were incubated in 0.1 mol l<sup>-1</sup> NH<sub>4</sub>Cl for 3 min, washed three times in PBS (5 min each), then in 0.1% Tween for 20 min and in 1% bovine serum albumin (BSA) for 10 min. After that, sections were incubated in mouse immunoglobulin G (IgG) monoclonal anti-tubulin (Sigma, T-9026) diluted 1:100 with PBS for 1 h at 37 °C. The samples were rinsed three times in PBS for 5–10 min each and then incubated in FITC-conjugated goat-anti-mouse IgG (Sigma, F-0257) diluted 1:100 with PBS for 1 h. The samples were rinsed three times in PBS for 5–10 min each and then stained with 1 µg ml<sup>-1</sup> PI (propidium iodide in PBS) for 1 min. The samples were again rinsed three times in PBS for 5–10 min each and then mounted in an anti-fade solution. A Leica SP2 laser scanning confocal microscope was used to scan the samples.

## Results

### Genotypes of parents and hybrids at *S-a*, *S-b* and *S-c* loci and pollen fertility of hybrids

The genotypes of parents and the hybrids at the *S-a*, *S-b*, and *S-c* loci were detected using closely linked molecular markers (Fig. 1) and are presented in Table 1. Parents have different alleles at different loci, so hybrids showed different pollen sterility gene interactions. Pollen fertility, seed set percentage, and alleles at the *S-a*, *S-b*, and *S-c* loci of five hybrids are shown in Table 2. Guanglu'ai 4-4xL202-4x and Guanglu'ai 4-4xJackson-4x have *S<sup>i</sup>* and *S<sup>j</sup>* alleles at the *S-b* locus, *S<sup>i</sup>* *S<sup>i</sup>* alleles at the *S-c* locus, and *S<sup>n</sup>* (neutral allele) and *S<sup>j</sup>* alleles at the *S-a* locus, and their pollen fertility and seed set was high. Guanglu'ai 4-4x×E24-4x has *S<sup>i</sup>* and *S<sup>j</sup>* alleles at the *S-b* and *S-c* loci, *S<sup>n</sup>* and *S<sup>j</sup>* at the *S-a* locus and its pollen fertility and seed set was low. L202-4x×E24-4X and Jackson-4x×E24-4x have *S<sup>i</sup>* and *S<sup>j</sup>* alleles at these three loci and had very low pollen fertility and seed set. These results indicated that pollen sterility loci interaction affected the pollen fertility of autotetraploid hybrids and pollen fertility further decreased with an increase in pollen sterility gene interactions.



**Fig. 1.** Parental genotypes at the *S-a*, *S-b*, and *S-c* loci detected with molecular markers G02-78.5, A07-55, and P24-85.7, respectively. 1–4 indicates parents (Guanglu'ai 4, L-202, Jackson, and E-24), 'i' and 'j' represents the *indica* and *japonica* genotypes, respectively; Guanglu'ai 4 has a neutral allele (*S<sup>n</sup>*) at the *S-a* locus (Long, 2007).

### Ultra-structural changes before and during PMC meiosis in autotetraploid rice hybrids

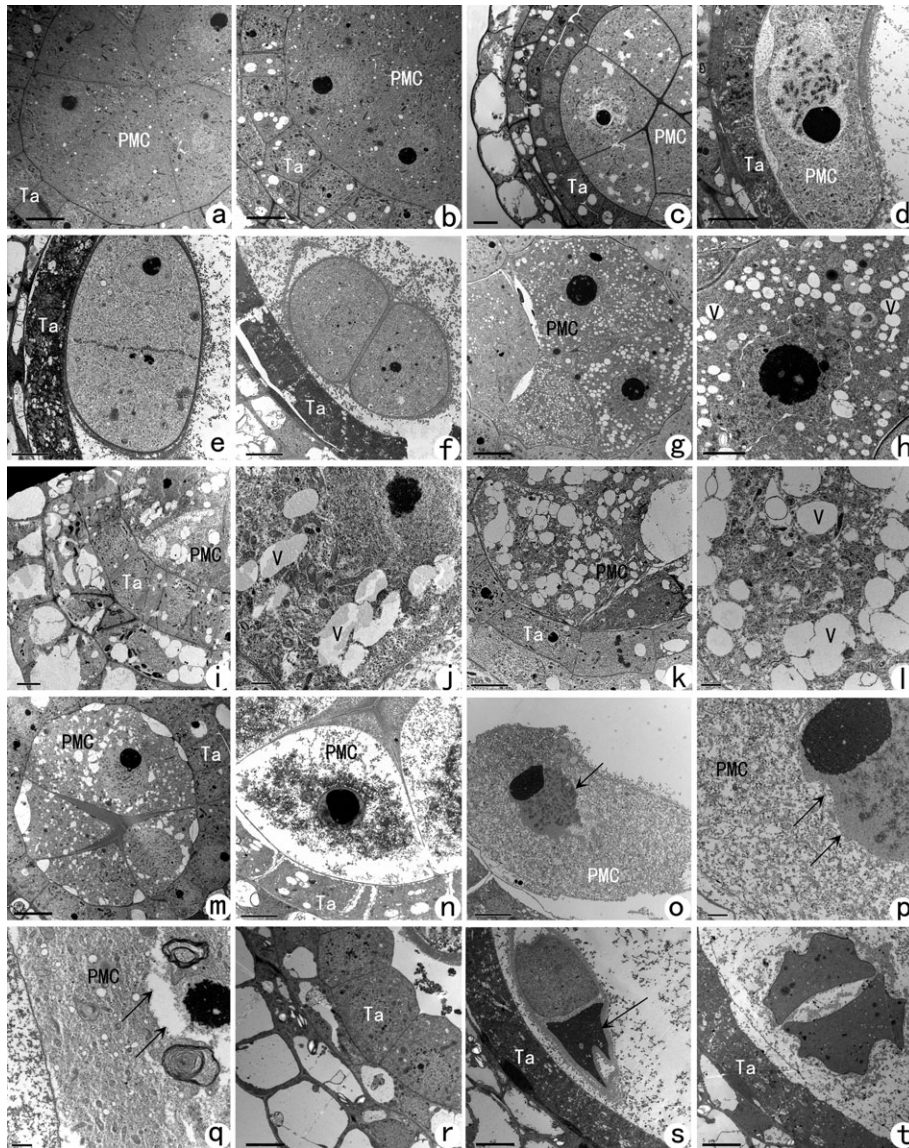
In order to understand the stage-time for pollen sterility gene interaction and its effect on pollen development, ultra-structural changes in PMC before and during meiosis were observed in the hybrids of autotetraploid rice. TEM (Transmission Electronic Microscopy) observation indicated that low seed-setting hybrids (L202-4x×E24-4x and Jackson-4x×E24-4x) displayed irregular ultra-structure, especially abnormalities in the cytoplasm and nucleus at the interphase-stage of PMC. High seed set hybrids produced normal ultra-structure similar to the control, i.e. small vacuolation was less and the structure of the organelles appeared normal in PMCs (Fig. 2a–f). Cytoplasmic abnormality was mainly characterized by excessive vacuolation in some PMCs. Vacuolation in these PMCs was relatively hard and plenty of vacuoles were observed in cells (Fig. 2g–m). The cytoplasm appeared thin, the organelles and the nuclear envelope (karyotheca) degraded gradually, and there was the formation of a compound membrane structure and nuclear vacuoles, the diffusion of karyoplasms, a gradually shrinking of the nucleolus, and, consequently, the degradation of PMCs (Fig. 2n–q). Organelles were pressed and distributed unevenly in PMCs, resulting in the asymmetric division of cytoplasm during meiosis which resulted in abnormal dyads (Fig. 2s) or tetrads (Fig. 2t). An abnormal tapetum was also observed in these PMCs (Fig. 2r). Ultra-structure abnormalities mostly occurred before meiosis and during meiosis prophase I in PMCs of the low-seed-set hybrids but less in high-seed-set hybrids, so it was presumed that these abnormalities were stimulated by pollen sterile gene interactions and was probably not directly related to chromosome behaviour in autotetraploid rice.

Microtubules were also abnormally distributed at leptotene during meiosis (Fig. 3b, d), at this stage, homologous chromosomes did not pair and, therefore, the abnormality was caused by gene interactions.

### Chromosome behaviours and configuration during PMC meiosis of intersubspecific autotetraploid rice hybrids

**Chromosome behaviour and configuration in PMC meiosis:** Intersubspecific autotetraploid rice hybrids with genetic interaction between different pollen sterility loci were used to observe PMC meiosis and chromosome behaviour, in order to prove if there is any difference in the chromosome behaviour of PMC meiosis and their relationship with pollen sterility loci. The results showed significant differences in chromosome configuration at diakinesis and metaphase I of the five hybrids (Table 3), though their meiosis processes were similar to that of diploid rice (Figs 3e–j, 4a).

Abnormal chromosome configuration, such as quadrivalents, trivalents, and univalents was found in the five hybrids (Fig. 4b–i). There were highly significant differences



**Fig. 2.** Ultra-structural changes of a pollen mother cell (PMC) before and during meiosis in the hybrids of autotetraploid rice. (a–d, f) Normal meiosis in diploid rice (CK). (g–t) Abnormal meiosis in the hybrids of autotetraploid rice with a low setting rate; Ta, tapetum; V, vacuole. Bars are 1  $\mu\text{m}$  in (j), (l), (p), and (q), 2  $\mu\text{m}$  in (h), and all other bars are 5  $\mu\text{m}$ . (a) Normal PMC at early interphase; (b) normal PMC at interphase; (c) normal PMC at leptotene; (d) normal PMC at pachytene; (e) normal dyad of Guanglu'ai 4-4x; (f) normal tetrad showing two cells. The other two cells are another section and are not shown. (g) Abnormal PMC at early interphase; vacuoles formed in the PMC; (h) magnification of (g) showing vacuoles in one PMC; (i) abnormal PMC at interphase showing many vacuoles in the PMC; (j) magnification of (i) showing the vacuoles in one PMC; (k) abnormal PMC at leptotene and plenty of vacuoles and abnormal organelles are present in the abnormal PMC; (l) magnification of (k) showing the vacuoles and abnormal organelles in the PMC; (m) leptotene showing the abnormal PMC; (n) zygotene with degraded PMC; (o) pachytene showing the abnormal nucleus of the PMC (arrow); (p) magnification of (o) and the nuclear membrane of PMC has degenerated (arrows); (q) pachytene where the nucleus and cytoplasm are both abnormal at the same time. Arrows indicate the abnormal nucleus of the PMC. (r) Abnormal proliferating tapetum. (s) Abnormal dyad, one has degenerated (arrow). (t) Abnormal tetrad.

among the five hybrids ( $F=8.67^{**}$ ) for quadrivalents and the average number of quadrivalents per cell ranged from 5.23 to 6.65 at diakinesis (Table 3). Hybrids with a low seed-setting rate had a greater number of quadrivalents per cell, whereas hybrids with a high seed-setting rate had a smaller number of quadrivalents per cell. The hybrid, Jackson-4x×E24-4x, had the highest number of quadrivalents per cell (6.65), Guanglu'ai 4-4x×L202-4x had the

lowest (5.23). More than six quadrivalents per cell were found in two hybrids with a low seed-setting rate which had genetic interaction of three pollen sterility loci. The main types of quadrivalents were: ring, chain, frying pan, Y shape, double ring shape, OK shape, x shape, -0- shape, and >-< shape during diakinesis (Table 4). Ring and chain shapes were the most frequent configurations of quadrivalents (Table 4). There was a significant difference

**Table 3.** Meiotic chromosome configurations at diakinesis and metaphase I in intersubspecific autotetraploid rice hybrids

Combinations	Stages	Chromosome configuration <sup>a</sup>
Guanglu'ai 4-4x×L202-4x	Diakinesis	(0.72±1.37)I+(13.00±3.59)II+(0.13±0.37)III+(5.23±1.85)IV
	Metaphase I	(1.06±1.42)I+(14.13±3.77)II+(0.20±0.41)III+(4.52±1.85)IV
Guanglu'ai 4-4x×Jackson-4x	Diakinesis	(0.23±0.64)I+(12.83±4.03)II+(0.08±0.27)III+(5.47±2.00)IV
	Metaphase I	(0.57±0.88)I+(14.40±3.87)II+(0.05±0.22)III+(4.62±1.87)IV
Guanglu'ai 4-4x×E24-4x	Diakinesis	(0.42±0.87)I+(10.70±3.81)II+(0.16±0.42)III+(5.30±1.93)IV
	Metaphase I	(0.92±1.31)I+(12.45±3.40)II+(0.24±0.47)III+(5.36±1.79)IV
L202-4x×E24-4x	Diakinesis	(0.59±1.06)I+(10.55±3.98)II+(0.10±0.31)III+(6.50±2.02)IV
	Metaphase I	(0.76±1.45)I+(10.89±4.22)II+(0.13±0.34)III+(6.27±2.09)IV
Jackson-4x×E24-4x	Diakinesis	(0.42±0.92)I+(10.26±3.31)II+(0.16±0.45)III+(6.65±1.66)IV
	Metaphase I	(0.96±1.09)I+(11.83±3.51)II+(0.46±0.62)III+(5.50±1.69)IV

<sup>a</sup> I, II, III, and IV represent univalent, bivalent, trivalent, and quadrivalent, respectively.

in the number of ring quadrivalents and a highly significant difference in the number of chain quadrivalents ( $F=6.06^*$ ,  $F=3.23^*$ ) among all the hybrids. Hybrids with the parent E24-4x had genetic interaction of two or three pollen sterility loci, and had more than five quadrivalents per cell.

Different configurations of quadrivalents with different numbers per cell were also found at metaphase I (Tables 3, 4). The number of quadrivalents clearly decreased in the hybrids with high pollen fertility (Guanglu'ai 4-4x×L202-4x and Guanglu'ai 4-4x×Jackson-4x). However, the hybrids with low pollen fertility, L202-4x×E24-4x and Jackson-4x×E24-4x, still had a high frequency of quadrivalents (Table 4), suggesting that more abnormalities would happen at the later stages with more abnormal pollen produced at maturity.

Guanglu'ai No.4-4x×E24-4x and Jackson-4x×E24-4x had the highest frequency (0.16 per cell) of trivalents, whereas Guanglu'ai No.4-4x×Jackson-4x had the lowest (0.08 per cell) at diakinesis (Table 4). There was insignificant difference for trivalents among the five hybrids.

Bivalents frequency was ranged from 10.26 to 13.00 per cell during diakinesis (Table 4). Hybrids with a low set have a lower frequency of bivalents than the higher seed set hybrids, but the bivalent types showed an insignificant effect on seed set. A higher number of ring bivalents was found than rod bivalents in all hybrids. Guanglu'ai No 4-4x×L202-4x had the highest number of bivalents, while Jackson-4x×E24-4x had the lowest number of bivalents. There was an insignificant difference in the number of univalents per cell at diakinesis among the five hybrids (Table 4).

#### Abnormal chromosome behaviour in PMC meiosis

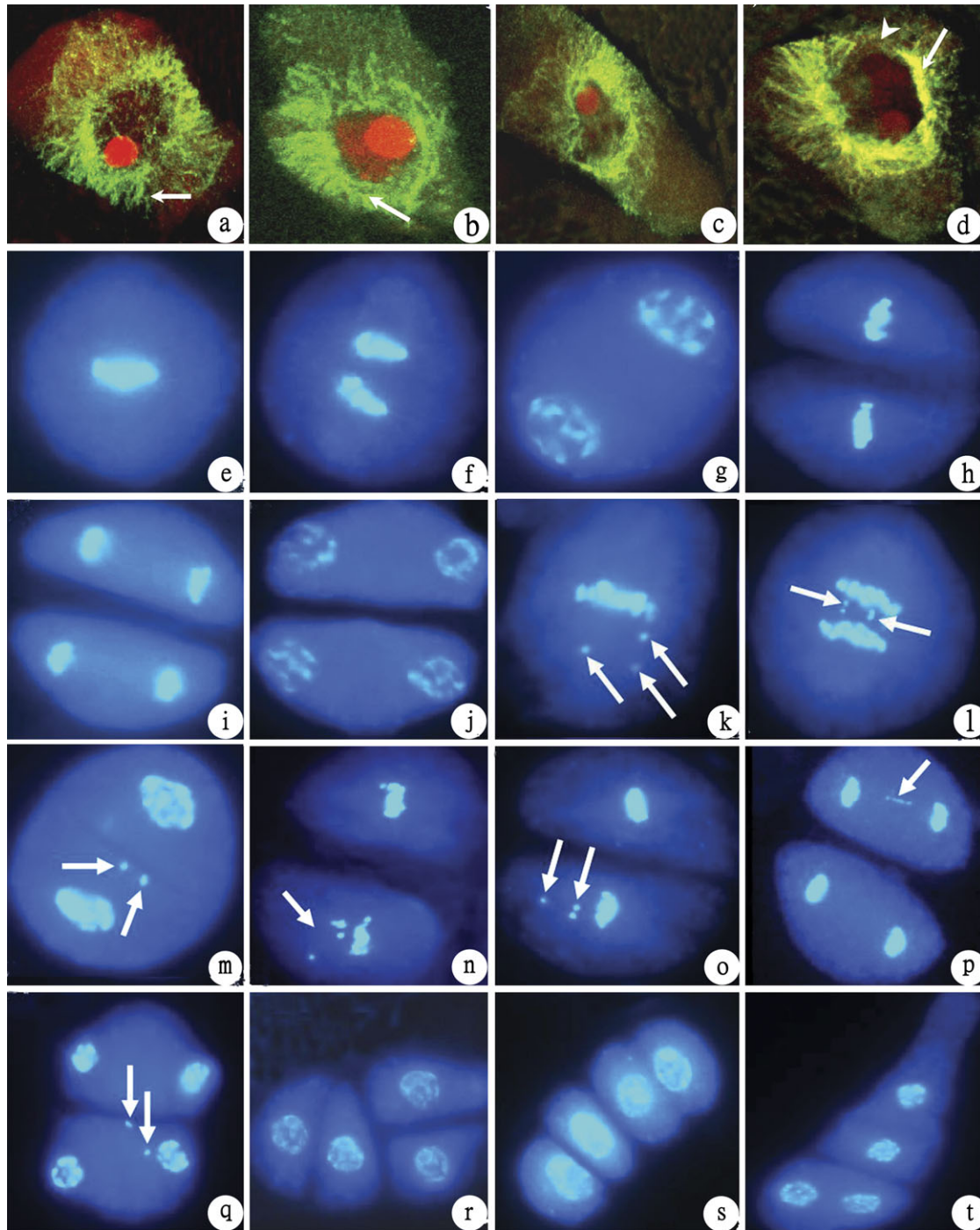
The various kinds of chromosomal abnormalities were found in PMC meiosis of low seed-set hybrids (Fig. 3k–t). Hybrids with genetic interaction of three pollen sterility loci showed more irregularities than hybrids with genetic interaction at just one pollen sterility locus. The main types of abnormalities include: chromosome straggling at metaphase I (Fig. 3k), chromosome lagging (Fig. 3l), and

a chromosome bridge at anaphase I; micro-nuclei at telophase I (Fig. 3m); chromosome straggling at metaphase II (Fig. 3n, o); abnormal spindle, asynchrony of chromosome disjunction, no separation or incomplete separation of two daughter cells, abnormal cell shape at metaphase II; chromosome lagging at anaphase II (Fig. 3p); two micronuclei at telophase II (Fig. 3q), asynchrony of chromosome separation, tri-fission, incomplete separation or no separation of cytokinesis, abnormal cell shape at telophase II; abnormal tetrad (Fig. 3r–t).

The average frequency of abnormalities at metaphase, anaphase, and telophase I were 24.03%, 27.11%, and 8.33%, respectively (Table 5). Among the five hybrids, the average frequency of abnormalities was higher at metaphase I (11.54%), anaphase I (6.91%), and telophase I (7.57%) in the two hybrids which had low seed-setting rates and genetic interaction of three pollen sterility loci. Hybrid L202-4x×E24-4x had the highest frequency of abnormalities (41.09% at metaphase I, 31.33% at anaphase I, and 18.29% at telophase I). The average frequency of abnormalities in the five hybrids at metaphase II, anaphase II, and telophase II was 30.03%, 24.76%, and 15.63%, respectively. L202-4x×E24-4x had the highest frequency of abnormalities at metaphase II, anaphase II, and telophase II (Table 5).

#### Relationship among chromosome configuration, abnormal chromosome behaviour, seed-setting rate, and pollen fertility

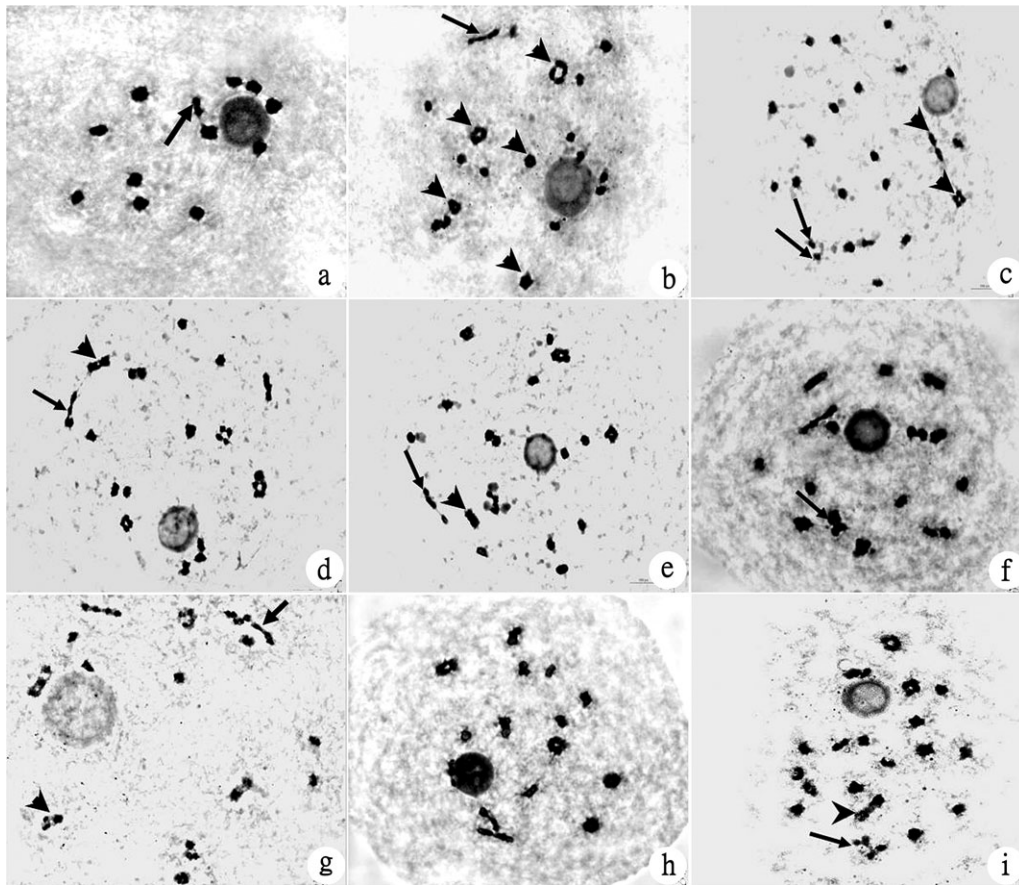
Correlation analysis showed that there was a highly significant and positive correlation between pollen fertility and seed setting rate ( $r=0.933^{**}$ ) (Table 6). This result showed that pollen fertility is one of the most important factors affecting the seed-setting rate of intersubspecific autotetraploid rice hybrids. Correlation analysis among chromosome configuration, pollen fertility, and seed-setting rate can be seen in Table 6. The total number of quadrivalents showed highly significant negative correlations with pollen fertility and seed setting rate ( $r=-0.232^{**}$ ,  $r=-0.197^{**}$ ). Among all kinds of quadrivalent configurations, pollen fertility and seed-setting rate depicted negative correlation with single ring quadrivalents and double ring quadrivalents. Trivalents



**Fig. 3.** Changes in the organization pattern of microtubules and chromosome behaviour during PMCs meiosis. (b–d) Microtubules of L202-4x×E24-4x; (e–j) chromosome behaviour of PMC meiosis in diploid rice (normal, control); (k–t) abnormal chromosome behaviour of PMC meiosis in the hybrid of L202-4x×E24-4x (×3000). (a) Leptotene, normal radiating microtubule bundles around the nuclear envelope (arrow) (Guanglu'ai 4-4x×L202-4x, control); (b) leptotene, abnormal radiating microtubules showing the short microtubule bundles (arrow); (c) leptotene, abnormal cell shape and microtubules; (d) leptotene, abnormal cell shape, nuclear envelope breaks down (arrowhead), and abnormal microtubules form an obvious envelope around the nucleus (arrow); (e) metaphase I; (f) anaphase I; (g) telophase I; (h) metaphase II; (i) anaphase II; (j) telophase I; (k) two straggling chromosomes (arrow); (l) lagging chromosome (arrow) at anaphase II; (m) two micronuclei at telophase I (arrows); (n, o) straggling chromosomes at metaphase II; (p) chromosome lagging at anaphase II (arrow); (q) two micronuclei (arrow) at telophase II; (r) abnormal tetrad; (s) tetrad with linear arrangement; (t) abnormal tetrad.

showed a significant and negative relationship with pollen fertility and seed set during metaphase I. Pollen fertility and seed set were highly significantly and positively correlated

with bivalents during diakinesis and metaphase I. Negative and non-significant relationship of seed set and pollen fertility with univalents was also observed.



**Fig. 4.** Chromosome configuration at diakinesis in the hybrids of autotetraploid rice ( $\times 3000$ ). (a) Chromosome configuration at diakinesis in diploid rice (control), 12II (arrow indicating rod-shaped chromosome); (b) 6IV [5ring shape (arrowhead)+1chain shape (arrow)]+12II (12ring shape); (c) 2IV (arrow head)+19II (17ring shape+2rod shape)+2I (arrow); (d) 7IV [3ring shape+1chain shape (arrow)+1 frying pan shape (arrowhead)+2 double rings shape]+10II (8ring shape+2rod shape);(e) 5IV [4ring shape+1 double rings shape (arrowhead)]+1III (chain shape, arrow)+12II (11ring shape+1rod shape)+1I; (f) 9IV [1 ring shape+2 chain shape+1 OK shape (arrow)+5 double rings shape]+6II (ring shape); (g) 5IV [1ring shape+1chain shape+1double rings shape+2 OK shape (arrowhead)]+1III (chain shape, arrow)+12II (10ring shape+2rod shape)+1I; h. 9IV (5ring shape+1 -0- shape+2 f.pan shape+1chain shape)+6II (5ring shape+1rod shape); (i) 4IV [2 ring shape+1 >> shape (arrowhead)+1 x shape (arrow)]+16II (14 ring shape+2 rod shape).

## Discussion

### *Allelic interaction is one of the most important reasons for low pollen fertility*

The cytological mechanism of pollen abortion is very complicated in intersubspecific autotetraploid rice hybrids and there are many factors associated with it (Xiao *et al.*, 2005; Zhao *et al.*, 2006; Hu *et al.*, 2009; Luan *et al.*, 2009). Although, it was speculated that irregular meiosis affected the fertility of hybrids along with some genes (Yan and Bao, 1960; Chen *et al.*, 1987), however, there was no direct evidence to prove this speculation. Our previous study indicated that allelic interaction of the  $F_1$  sterility loci is the major reason for intersubspecific hybrid sterility in diploid rice and these sterility alleles could also cause intersubspecific hybrid sterility in autotetraploid rice (Zhang *et al.*, 2006; Zhao *et al.*, 2006). In this study, five autotetraploid rice lines with different pollen sterility loci were used and hybrids made with different pollen allelic interaction. The results demonstrated that interaction of the pollen sterile

loci may result in partial or complete sterility of the intersubspecific autotetraploid rice hybrids and indicated that, as the number of  $S^i$  and  $S^j$  alleles increased at different loci in the autotetraploid hybrids, their pollen fertility decreased significantly; hybrids with three pairs of  $S^i$  and  $S^j$  alleles had very low pollen fertility and seed set. These results are consistent with the findings of Zhang *et al.* (2006) in that they also reported the partial abortion of male gametes caused by the interaction between  $S^i$  and  $S^j$  alleles in diploid rice. Moreover, our results also proved that the neutral allele ( $S^n$ ) did not interact with  $S^i$  and  $S^j$  alleles and autotetraploid hybrids displayed normal pollen fertility with the neutral alleles. Therefore, our results suggest that allelic interaction between  $S^i$  and  $S^j$  alleles caused pollen abortion which, in turn, reduced the seed set of autotetraploid hybrids and, on the other hand, also suggest that neutral alleles may have the potential to overcome hybrid sterility. So far, some new lines or hybrids with normal pollen fertility and seed set have been developed in our laboratory by using materials with the neutral alleles of pollen fertility.



**Table 4.** Frequencies of different chromosome configurations and shapes at diakinesis and metaphase I in intersubspecific autotetraploid rice hybrids

Materials	Stage <i>n</i> <sup>c</sup>	Quadrivalents per cell							Trivalents per cell			Bivalents per cell			Univalents per cell	Chiasma per cell	
		Ring	Chain	F.pan	Y	Double rings		Total	Chain	F.pan	Total	Rod	Ring	Total			
						Others	Total										
Guanglu'ai 4-4x×L202-4x	Diak <sup>a</sup>	88	4.28	0.59	0.14	0.05	0.10	0.07	5.23	0.06	0.07	0.13	3.74	9.26	13.00	0.72	42.51
	M-I <sup>b</sup>	54	3.81	0.37	0.13	0.15	0.00	0.06	4.52	0.17	0.04	0.20	4.61	9.52	14.13	1.06	41.13
Guanglu'ai 4-4x×Jackson-4x	Diak	64	4.53	0.63	0.11	0.02	0.16	0.03	5.47	0.06	0.02	0.08	3.27	9.57	12.83	0.23	43.30
	M-I	58	4.24	0.16	0.07	0.00	0.12	0.04	4.62	0.05	0.00	0.05	3.12	11.27	14.40	0.57	44.03
Guanglu'ai 4-4x×E24-4x	Diak	116	5.30	0.65	0.27	0.06	0.09	0.06	5.30	0.09	0.07	0.16	1.81	8.89	10.70	0.42	44.91
	M-I	127	5.05	0.19	0.12	0.01	0.00	0.00	5.36	0.12	0.13	0.24	3.35	9.10	12.45	0.92	43.42
L202-4x×E24-4x	Diak	116	5.20	0.72	0.18	0.05	0.29	0.06	6.50	0.07	0.03	0.10	3.45	7.10	10.55	0.59	43.18
	M-I	45	5.18	0.58	0.16	0.02	0.27	0.07	6.27	0.09	0.04	0.13	3.31	7.57	10.89	0.76	43.16
Jackson-4x×E24-4x	Diak	31	5.68	0.45	0.19	0.10	0.23	0.00	6.65	0.06	0.10	0.16	2.45	7.81	10.26	0.42	44.48
	M-I	48	4.90	0.19	0.10	0.00	0.31	0.00	5.50	0.38	0.38	0.46	3.46	8.38	11.83	0.96	43.33
Average	Diak		5.00	0.61	0.18	0.06	0.17	0.04	5.83	0.07	0.06	0.13	2.94	8.53	11.47	0.48	43.68
	M-I		4.64	0.30	0.12	0.04	0.14	0.03	5.25	0.16	0.12	0.22	3.57	9.17	12.74	0.85	43.01

<sup>a</sup> Diakinesis.  
<sup>b</sup> Metaphase I.  
<sup>c</sup> Number of PMCs observed.

**Table 5.** Frequency of abnormal chromosome behaviors during meiosis of intersubspecific autotetraploid rice hybrids

Materials	Meiosis I						Meiosis II					
	Metaphase I		Anaphase I		Telophase I		Metaphase II		Anaphase II		Telophase II	
	<i>n</i> <sup>a</sup>	Abnormal cells (%)	<i>n</i> <sup>a</sup>	Abnormal cells (%)	<i>n</i> <sup>a</sup>	Abnormal cells (%)	<i>n</i> <sup>a</sup>	Abnormal cells (%)	<i>n</i> <sup>a</sup>	Abnormal cells (%)	<i>n</i> <sup>a</sup>	Abnormal cells (%)
Guanglu'ai 4-4x×L202-4x <sup>b</sup>	400	16.79	343	17.82	763	6.65	172	24.87	86	24.89	122	18.85
Guanglu'ai 4-4x×Jackson-4x	235	17.45	91	19.78	66	15.15	106	17.92	158	14.56	236	8.05
Guanglu'ai 4-4x×E24-4x	348	18.68	67	13.43	23	4.35	236	21.19	100	16.00	17	5.88
Average		17.64		17.01		8.72		21.33		18.48		10.93
L202-4x×E24-4x <sup>b</sup>	183	41.09	83	31.33	315	18.29	256	47.38	68	27.94	86	33.72
Jackson-4x×E24-4x	898	17.27	161	17.83	101	12.73	270	31.93	82	20.57	87	25.29
Average		29.18		24.58		15.51		39.66		24.26		29.51
Total average		24.03		27.11		8.33		30.03		24.76		15.63

<sup>a</sup> Number of PMCs observed.  
<sup>b</sup> Some data cited from the result of He *et al.* (2011).

*Relationship between pollen sterility interaction of alleles, and abnormal chromosome configuration and their effect on the fertility of intersubspecific autotetraploid hybrids during meiosis*

Sterile aneuploid gamete frequency depends on the separation of homologous chromosomes with different associations in autotetraploidy, because quadrivalents are the main chromosome association and its way of separation is the most important factor. The way of quadrivalent separation depends on the chromosome configuration. There were 11 types of quadrivalent configurations found in autotetraploid plants in diakinesis and metaphase I (Kuspira and Bhamhani, 1984),

while in autotetraploid rice, three main kinds of quadrivalent configurations could be found, i.e. ring, chain, and frying pan shapes (Luan *et al.*, 2007). A variety of quadrivalents was also found, such as double rings, -0- shape, Y shape, OK shape, x-shape, and >-<shape, along with the three main types, in autotetraploid rice. All these configurations appeared in different ratios. A higher frequency of bivalents and quadrivalents and a smaller number of trivalents and univalent was found. Quadrivalents were the most frequent in all autotetraploid plants (70–100% depending on the plant) and ring-shaped quadrivalents were more common than other shapes (Espinasse *et al.*, 1995). Similarly, ring-shaped quadrivalents and bivalents were found more frequently in the present study

**Table 6.** Correlation analysis among chromosome configurations and chiasma at diakinesis and metaphase I, seed setting rate and pollen fertility of intersubspecific autotetraploid rice hybrids

Chromosome configuration		Diakinesis <sup>a</sup>			Metaphase I <sup>a</sup>		
		Chiasma	Pollen fertility	Seed setting rate	Chiasma	Pollen fertility	Seed setting rate
Quadrivalents	Ring	0.421**	-0.192**	-0.146**	0.462**	-0.163**	-0.141*
	Chain	-0.240**	-0.001	-0.023	-0.165**	-0.052	-0.135*
	F.pan	0.153**	-0.043	-0.014	0.095	-0.021	-0.030
	Double rings	0.186**	-0.132**	-0.160**	0.196**	-0.278**	-0.313**
	Total	0.350**	-0.232**	-0.197**	0.434**	-0.216**	-0.230**
Trivalents	Chain	-0.203**	0.004	0.013	-0.292**	-0.213**	-0.156**
	F.pan	-0.019	-0.019	0.008	-0.008	-0.016	0.019
	Total	-0.162**	-0.012	0.012	-0.247**	-0.187**	-0.118*
Bivalents	Ring	0.174**	0.232**	0.261**	-0.788**	0.239**	0.253**
	Rod	-0.784**	0.056	-0.048	0.191**	0.038	0.045
	Total	-0.278**	0.235**	0.203**	-0.299**	0.238**	0.248**
Chiasma		—	-0.052	0.041	—	-0.050	-0.036
Univalents		-0.483**	-0.020	-0.040	-0.563**	-0.034	-0.006

<sup>a</sup> \* Significance at 0.05 probability level; \*\* significance at 0.01 probability level.

compared with other quadrivalent types at diakinesis and Metaphase I of PMCs. Many bivalents and a few trivalents and quadrivalents were observed at diakinesis or Metaphase I of PMCs in tetraploid *C. persicum* and *C. graecum*, and ring bivalents were most frequent at Metaphase I of PMCs (Ishizaka, 2003). By contrast, Luan *et al.* (2009) reported a greater number of rod bivalents than ring bivalents in autotetraploid restorer lines.

There are few reports related to the relationship between chromosome configuration and fertility in autotetraploid plants. Multivalent formation in polyploids is usually negatively correlated with fertility and the tendency of many tetraploids towards low quadrivalent frequencies and high bivalent frequencies has significant importance in biological implications and practical applications (Jones, 1994). A lower frequency of quadrivalents resulted in a higher pollen fertility and seed set in our autotetraploid hybrids. These results are in agreement with previous reports that low quadrivalents resulted in high fertility of autotetraploid rye (Jenkins and Chatterjee, 1994) and tetraploid potato (Watanabe *et al.*, 1994), however, there have been contradictory statements that higher fertility was achieved due to a higher number of quadrivalents in tetraploid *Lolium* (Crowley and Rees, 1968) and autotetraploid rice (Luan *et al.*, 2009).

Our results showed that there was a significant difference among autotetraploid hybrids, especially, among the hybrids with one pollen sterile gene interaction, which had high pollen fertility, and those with three loci interaction, which had low pollen fertility. As for the reason for the difference in the number of quadrivalents, it is thought that it might be related to pollen sterility loci interaction because more quadrivalent configurations (such as ring and chain shapes) were observed in the hybrids with low pollen fertility than in those with high pollen fertility. It requires further studies to reveal that how pollen sterility loci interaction affect the chromosome configuration.

#### *Relationship between pollen sterility alleles interaction and abnormal chromosome behaviour and their effect on pollen fertility during meiosis in autotetraploid rice*

Chromosome differentiation may play an important role in the preferential pairing behaviour of polyploids when homologous partners are contributed by less related parental lines. Competition between preferential chromosome association and potential partner may affect synapsis and chromosome recombination and thus affect the meiotic process and the pollen fertility of colchicine-induced autotetraploid hybrids (Benavente and Orellana, 1991). Reproductive stability and fertility in induced autotetraploids of kale depends on the precise control of chromosome pairing at meiosis (Jenczewski *et al.*, 2002). High pollen fertility and normal seed setting are features of normal meiotic chromosome division (Srivastava *et al.*, 1992; Majumdar *et al.*, 2004; Adamowski *et al.*, 2008; He *et al.*, 2011). Pollen viability and plant fertility are directly related to the meiotic abnormalities stimulated by defective chromosome pairing, unequal chromosome distribution or non-disjunction (Luo *et al.*, 1992; Singh, 1992). Similar results were also reported in the present study that chromosome behaviour has a highly significant relationship with pollen fertility and seed set in autotetraploid rice.

In our study, many abnormal chromosome behaviours were observed in the hybrids with low pollen fertility. As the allelic interaction of F<sub>1</sub> pollen sterility loci increased, chromosomal abnormalities also increased, which resulted in the low pollen fertility of autotetraploid rice. Some differences were found in pollen fertility, seed-setting rate, and abnormal chromosome behaviour among hybrids derived from the same paternal or maternal parent. The mean frequency of abnormal chromosome behaviour was highest among hybrids whose male parent was E24-4x and the female parent was L202-4x, while a high frequency of

abnormal chromosome behaviour was found at metaphase and telophase II in the hybrid whose male parent was Jackson-4x. Therefore, it is supposed that the abnormal chromosome behaviour might be related to the genotype of the parents, i.e. related to the allelic interaction of F<sub>1</sub> pollen sterility loci. In other words, pollen sterility loci interaction (*S-a*, *S-b*, and *S-c*) lead to increased chromosomal abnormalities and result in the lower pollen fertility of autotetraploid rice hybrids.

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