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REVIEW

Development and characterization of synthetic amphiploid (AABB) between *Oryza sativa* and *Oryza punctata*

Aiyun Wang · Xianhua Zhang · Chunhua Yang · Zhaojian Song · Chaoqun Du · Dongling Chen · Yuchi He · Detian Cai

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Abstract *Oryza punctata*, a wild relative of cultivated rice, belongs to the BB genome of *Oryza*. Interspecific hybrids (CW008, AB) between *Oryza sativa* ($2n = 24$ AA) and *O. punctata* ($2n = 24$ BB) were obtained using embryo rescue technique. Synthetic allopolyploid (DCW008, AABB) were produced through chromosome doubling by colchicine. Hybrids overcame many wild traits except the shattering and awn. The synthetic amphiploid plants showed obvious superiority in growth and production. Interspecific hybrids CW008 were completely infertile but DCW008 had better seed set after selfing. Genomic in situ hybridization investigations were performed on DCW008. The result indicated that the A genome was closed to the B genome and translocations occurred between some chromosomes of cultivated and wild rice. Meiosis was nearly normal in the amphiploid hybrid but was disrupted in the diploid hybrid, which resulted in different fertility of them.

Keywords *Oryza punctata* · Amphiploid · Distant hybridization · GISH · Introgression

Abbreviations

BA	6-Benzylamino purine
CTAB	Hexadecyl trimethyl ammonium bromide
GA3	Gibberellin A3
MS	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
N6	N6 medium
2,4-D	2,4-Dichlorophenoxy acetic acid

Introduction

Rice is one of the world's three major food crops, providing a major source of food for more than 60 % of the world population. Faced on the pressure of a growing world population, the decrease in available arable land and the increasing quality of life, it has become important to increase rice yield and quality. However, the genetic resources of cultivated rice

Xianhua Zhang and Aiyun Wang contributed equally to this study. All research had been completed in Hubei University, China.

A. Wang · X. Zhang · Z. Song · D. Chen · Y. He · D. Cai (✉)
Faculty of Life Science, Hubei University, Wuhan 430062, People's Republic of China
e-mail: caidt8866@yahoo.com.cn

Present Address:
A. Wang
Faculty of Life Science, Ludong University, Yantai 264025, Shandong, People's Republic of China

C. Yang
University for Science and Technology Beijing, Beijing 102206, People's Republic of China

C. Du
Forestry Science Research Academy of Hubei Province, Wuhan 430075, People's Republic of China

have become relatively limited and the task of super rice breeding has become extremely difficult. Therefore, wild rice with rich genetic resources has become very important in rice breeding (He 1998; Deng et al. 2006; Zhu et al. 2008). The wild species are sources of useful genes for resistance to major disease and insects and for tolerance to abiotic stresses (Multani et al. 1994). Some useful genes have successfully been transferred from wild rice into cultivated rice, such as the gene for resistance to the grassy stunt virus from *Oryza nivara* (Khush 1977), cytoplasmic male sterility from *O. sativa f. spontanea* (Lin and Yuan 1980), bacterial blight resistance gene *Xa21* from *Oryza longistaminata* (Khush et al. 1990), rice blast resistance gene *pi9* from *Oryza minuta* (Amante-Bordeos et al. 1992). The genus Oryza consists of 22 wild species which have been certified, including AA-genome and non-AA-genome species. Cultivated rice and AA-genome wild species can be easily crossed. So, useful genes from the AA-genome wild species can be transferred into rice through conventional hybridization and backcrossing procedures. But hybridization between cultivated rice and non-AA-genome wild species are very difficult. The serious interspecific reproductive barriers make it difficult to transfer useful genes from the non-AA-genome wild species to cultivated rice (Zhong et al. 2000). Although with the development of the tissue culture technique, some interspecific hybrids have been developed and some useful genes from non-AA-genome wild species have been transferred into cultivated rice, the efficiency is still low. Polyploid rice with more genome has good plasticity. So, they can play a bridge role in the distant hybridization and promote the utilization of useful genes from wild species (Huang et al. 2001; Carvalho et al. 2005). To date, some artificial allopolyploidizations of *Oryza* have been created. A part of them had seed set after selfing (Wang et al. 2005). In this study, interspecific hybrids (AB) between *O. sativa* and *O. punctata* (BB genome) were obtained through embryo rescue. Amphiploid intergenomic hybrids (AABB) were produced through chromosome doubling by colchicine. The genomic components of the hybrid were verified by using GISH. A comparison and analysis between the diploid and synthetic amphiploid were undertaken for the main agronomic traits, pollen fertility and meiosis behavior.

Materials and methods

Plant materials

The cultivated rice Sgdts96, HN2026, Nipponbare (*O. sativa*, AA, $2n = 2x = 24$) were japonica rice lines stored at the Polyploid Genetics Lab of Hubei University, Wuhan, China. Wild rice *O. punctata*, (BB, $2n = 2x = 24$, IRRI Number: 105980) was kindly provided by the International Rice Research Institute (IRRI), Manila, Philippines. The diploid hybrid CW008 (AB, $2n = 2x = 24$) of Sgdts96 × *O. punctata* and synthetic amphiploid DCW008 (AABB, $2n = 4x = 48$) were used in this study.

Crosses and chromosome doubling

The cultivar Sgdts96, HN2026 and Nipponbare were used as the female parents in crosses with *O. punctata*. In order to promote early flowering in wild rice, *O. punctata* was dark treated, using a short photoperiod of 8 h per day, for 30 days from 10 June to 9 July 2004 in Wuhan, China. After pollination, hormone treatment with 2, 4-D (10–30 mg l^{-1}) and GA_3 (50–100 mg l^{-1}) was necessary. The immature hybrid embryos were excised and cultured on N6 solid medium ($N6 + 6BA_{0.5-2mg^{-1}\,l} + NAA_{0.5-2mg^{-1}\,l} + Sucrose_{5\%} + Agar_{0.75\%}$). For approximately 3 weeks, the shoots were transferred to a liquid medium with colchicine (500–750 mg^{-1} l) for chromosome doubling. 2 days later, they were shifted back to N6 medium. The shoots were rooted on 1/2 MS medium with 0.5 mg^{-1} l BA and 0.3 mg^{-1} l NAA. Plantlets with roots were transferred to the field.

Cytological observation and genomic in situ hybridization (GISH)

Root tips from F_1 and doubled plantlets were used for determination of the chromosome numbers. Directly-fixed flower buds were used for meiotic behavior observation (Li et al. 1995). Pollen fertility was determined as the percentage of pollen grains stained with 1 % I_2-KI.

For GISH, root tip cells were digested at 28 °C for about 4 h in an enzyme mixture containing 2 % cellulase and 2 % pectinase. Chromosome preparation mainly followed the method described by Yan et al. (1998) with some modifications. Total genomic DNA

of *O. sativa* Sgdts96 and *O. punctata* was extracted from young leaves using CTAB. The DNA of *O. punctata* was fluorescently labeled with bio-11-dUTP using nick translation, according to the manufacturer's instructions (Sino-American Biotechnology Company), and used as the probe. The genomic DNA from Sgdts96 was sheared by autoclaving for 5 min and used as the block. In situ hybridization was carried out according to the method of Leitch et al. (1990).

Comparison of morphology

The main morphological traits of diploid and amphiploid hybrids were compared. Recording methods and standards were set according to the protocols of Gai (1996).

Results

The construction of diploid and amphiploid intergenomic hybrids

There was a very low success rate for natural hybridization between cultivated rice and wild rice. The key to success for the wide cross is to overcome the problem of non-synchronization of flowering and cross-incompatibility. Currently, a short-day treatment was used to overcome the first problem. Principally it harmonizes flowering time between *O. sativa* and *O. punctata* at the same time. Then, hormone treatment and a combination of embryo rescue were used. Finally, intergenomic hybrids of *Oryza sativa* × *O. punctata* were obtained (Fig. 1). Statistical data for the fertility of different cross combinations are listed in Table 1.

The hybrid shoots were cultured on the medium with 500–750 mg^{-1} l colchicine for chromosome doubling. The total number of synthetic amphiploid plants which were cytological confirmed was 10. And the doubling frequency was 9.09–14.81 % (Table 2). Root tip cells' mitotic metaphase from the doubled plants with $2n = 48$ revealed the success of chromosome doubling.

Cytological and GISH analyses

The chromosome numbers in root tips of diploid and amphiploid plants were counted. As expected,

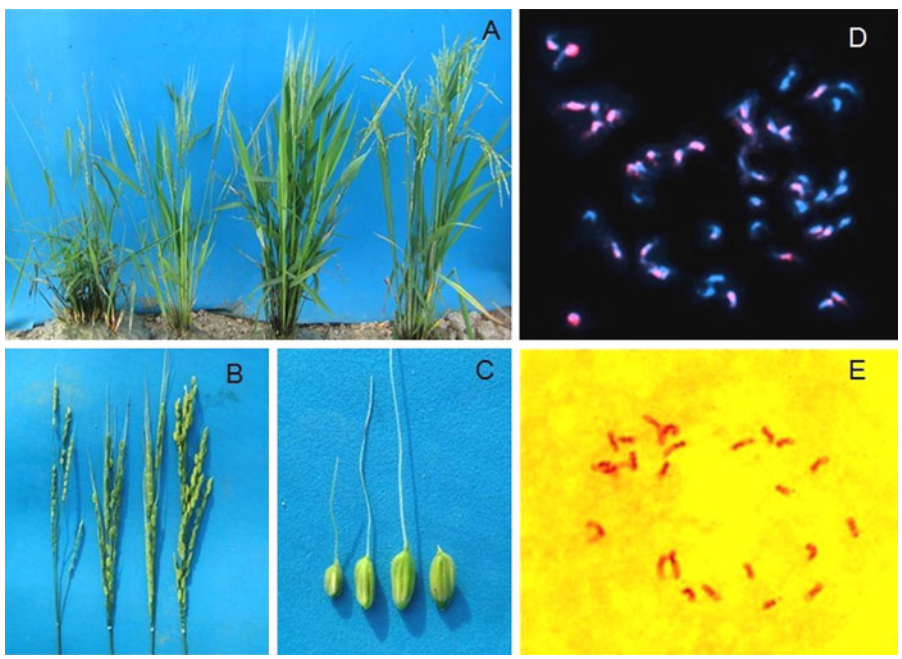

Fig. 1 *O. sativa*, *O. punctata* and their hybrids. **a** plants (*left* → *right*): *O. punctata* (male parent); diploid hybrid; amphiploid hybrid; *O. sativa* Sgdts96 (female parent); **b** panicles (*left* → *right*): *O. punctata*; diploid hybrid; amphiploid hybrid; *O. sativa* Sgdts96; **c** grains (*left* → *right*): *O. punctata*; diploid hybrid; amphiploid hybrid *O. sativa* Sgdts96; **d** GISH detection of amphiploid hybrid DCW008 (AABB, $2n = 4x = 48$); **e** Chromosomes of intergenomic hybrid CW008 (AB, $2n = 2x = 24$)

Table 1 The fertility of interspecific crosses between *O. sativa* × *O. punctata*

Hybrid combination	Genome	No. Of spikelets pollinated	No. of seeds	Seed set (%)	No. of embryos cultured	No. of embryo germination	Percentage embryo germination (%)
Sgdts96 × *O. punctata*	AB	106	11	10.38	11	1	9.09
HN2026 × *O. punctata*	AB	508	19	3.74	19	4	21.05
Nipponbare × *O. punctata*	AB	219	13	5.94	13	3	23.08

Table 2 Results of chromosome doubling

Hybrid combination	No. of treated shoots (D)	No. of regenerated plants	No. of tetraploid (T)	T/D %
Sgdts96 × *O. punctata*	27	24	4	14.81
HN2026 × *O. punctata*	29	21	3	10.34
Nipponbare × *O. punctata*	33	30	3	9.09

chromosomes of the intergenomic hybrid CW008 were $2n = 2x = 24$, and the synthetic amphiploid DCW008 were $2n = 4x = 48$ (Fig. 1d, e).

GISH investigations were performed on DCW008. GISH analyses indicated that 16 chromosomes were wholly labeled by the *O. punctata* probe. The other 11 chromosomes were partly labeled. We further investigated those partly labelled chromosomes. One arm was labeled on 2 chromosomes. And the chromosomal fragment attached to the terminal parts of 4 chromosomes. The other chromosomes' labeled fragments were irregular (Fig. 1d).

Pollen fertility test results

Pollen was stained by I_2-KI. The mature and normal pollen grains were full, round and dark. The aborted pollen grains could not be stained. Generally, the abortive pollen grains were irregular-shaped (small or triangular), and some had a round shape but no contents (Fig. 2). The staining rate of F_1 plants from the cross of *O. sativa* and *O. punctata* was nearly 0 %, while the staining rate of the amphidiploid was as high as 62.86 %.

Meiotic behavior of diploid and amphiploid hybrids

The diploid F_1 hybrids generally showed irregular meiosis, with mostly univalents. Of the 93 pollen mother cells (PMCs) observed, 58.51 % had more than 12 I and there was no quadrivalent (Table 3). The meiotic prophase I of the most PMCs from amphiploid hybrid had more than 18 II and sometimes up to 24 II. Of the 99 PMCs, 57.58 % showed 22–24 II. 2–3 quadrivalents association was recorded in 31.31 % of the cells. Univalent hasn't been observed in amphiploid hybrid.

Through the observation of meiotic metaphase I and anaphase I of the PMCs, we found that the percentage of lagging chromosomes in the diploid hybrid's PMCs reached 34.90 %. The ratio with three or more lagging chromosomes of PMCs was 6.93 % (Table 4). However, more than 92.71 % of the PMCs in the synthetic amphiploid DCW008 were normal. The percentage of cells seen with lagging chromosomes was only 7.29 % of the total number of PMCs. Generally, there was only one lagging chromosome. Two lagging chromosomes only appeared in 1.04 % of the PMCs. The result further demonstrated that meiosis had a great impact on fertility. Pollen mother cells from the diploid hybrid had a small number of bivalents and chromosome pairing disorders. In meiotic anaphase I, there appeared a large number of lagging chromosomes (Fig. 3b–c). They could not form normal gametes, which led to a high degree of infertility. By chromosome doubling, each chromosome paired up with another homologous chromosome, which normalized meiosis (Fig. 3d–e), thereby increasing fertility.

Agronomic traits of diploid and amphiploid hybrids

Compared to the *O. punctata* parent, the hybrids overcame many wild traits except the shattering and awn traits. The synthetic amphiploid plants had more sturdy stems and larger leaves and grains. In addition, the amphiploid plants showed stronger growth and tillering (Fig. 4a–c). The main agronomic traits

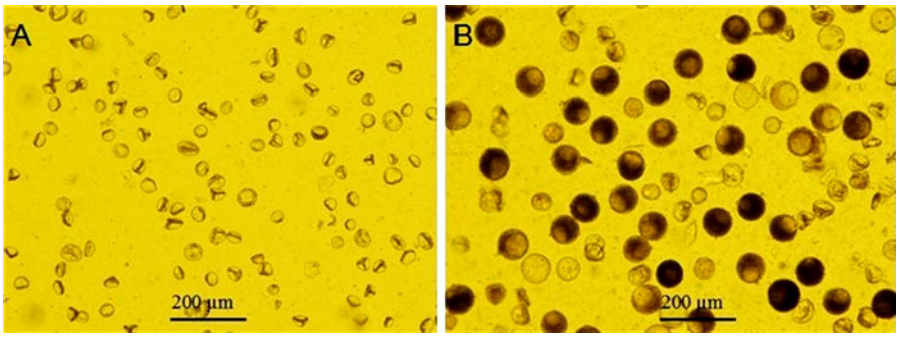

Fig. 2 Pollen staining pictures of diploid hybrid and amphiploid. **a** Abortive pollen from diploid hybrid CW008, **b** many stained pollen grains from amphiploid DCW008

Table 3 Observations of meiotic prophase I of the intergenomic diploid and amphiploid hybrids

Hybrid	Number of PMCs	Univalent/cell (no.) (no.)		Bivalent/cell (no.)		Quadrivalent/cell (no.)		Percentage (%)
		Mean	Range	Mean	Range	Mean	Range	
CW008 (2x)	19	19.26	18-22	2.37	1-3	/	/	20.21
	36	14.22	12-16	4.89	4-6	/	/	38.30
	33	8.18	6-10	8.52	7-9	/	/	35.12
	5	3.60	10-11	10.20	2-4	/	/	5.32
DCW008 (4x)	57	/	/	22.37	22-24	0.82	0-1	57.58
	31	/	/	19.42	18-20	2.29	2-3	31.31
	11	Others						11.11

I univalent, II bivalent, IV quadrivalent

Table 4 Lagging chromosomes of the meiotic metaphase I and anaphase I of diploid and tetraploid hybrids

Hybrid	Lagging chromosomes	Number of PMCs	Percentage (%)
CW008 (2x)	0	47	66.10
	1	10	13.89
	2	10	14.08
	≥3	5	6.93
DCW008 (4x)	0	89	92.71
	1	6	6.25
	2	1	1.04

showed no obvious character segregations in F_1–F_3 generations, which were relatively uniform (Fig. 4d–f). The diploid hybrids were completely infertile but amphiploid plants had good seed set after selfing, with some variations in seed setting (31.52–37.65 %) (Table 5). After several years' selection, the seed setting rate of some intergenomic amphiploid hybrid plant lines can reach about 60 %.

Discussion

Gene introgression from wild rice

Wild rice has many useful genes for traits, which involve the function of insect resistance, disease resistance, stress tolerance, high yield and high grain quality. Gene introgression is generally used to transfer useful genes from wild species into rice. Many genes from wild species have been transferred into cultivated rice through the introgression method. But gene introgression in diploid level has limitations. First, in the genus *Oryza*, interspecific hybridization is normally characterized by low seed set (0–26.5 %) that is commonly less than 10 % (Sitch et al. 1989a). And especially when involving intergenomic crosses, the hybrids are completely sterile. In this study, crosses between three varieties of *O. sativa* as the female parents and *O. punctata* as male parent gave seed sets between 3.74 and 10.38 %. Second, the diploid F_1 hybrids are usually completely sterile. In

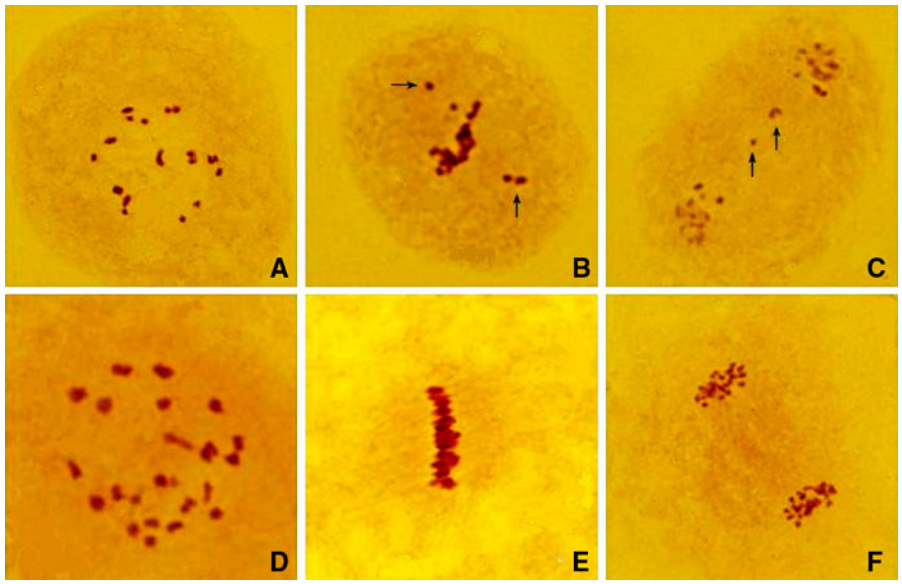

Fig. 3 Meiosis I of diploid and amphidiploid intergenomic hybrids of *O. sativa* × *O. punctata*. **a–c** Meiosis I of diploid hybrid (**a** prophase I, **b** metaphase I, **c** anaphase I), **d–f** meiosis I of amphiploid hybrid (**d** prophase I, **e** metaphase I, **f** anaphase I) (The *arrows* indicate the lagging chromosomes)

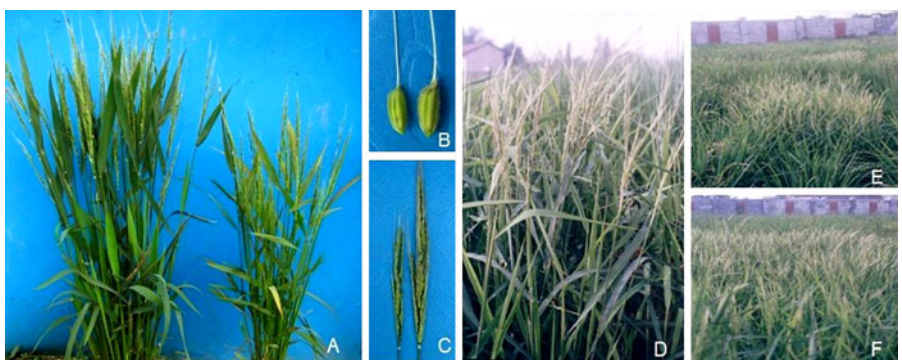

Fig. 4 Morphology of diploid and amphiploid hybrids. **a** Plants (*left* amphiploid, DCW008; *right* diploid, CW008), **b** grains (*left* diploid, CW008; *right* amphiploid, DCW008), **c** panicles (*left* diploid, CW008; *right* amphiploid, DCW008), **d** DCW008 F_1, **e** DCW008 F_2, **f** DCW008 F_3

this investigation, pollen fertility of diploid F_1 hybrids of *O. sativa* and *O. punctata* was 0 %. There were also non-functional female gametes produced by diploid F_1 hybrids. Mariam et al. (1996) approved that the sterility could be due to differences in the structure and number of chromosomes, a lack of chromosomal homology that results in a variable number of univalents and the production of unbalanced gametes that leads to high pollen abortion. Multani et al. (2003) reported that crossability of the F_1 hybrids of *O. sativa* and wild rice was extremely low and no seeds were obtained upon backcrossing.

However, when interspecific diploid hybrids doubled into allopolyploid by colchicine, their fertility can be partly restored. They can produce functional female gametes and have good seed set after selfing. As shown in this study, synthetic allopolyploid DCW008 was fertile. There are many allopolyploid wild rice species in nature, such as *O. minuta* (BBCC), *O. malampuzhaensis* (BBCC), *O. alta* (CCDD), *O. grandiglumic* (CCDD), *O. latifolia* (CCDD), *O. longiglumis* (HHJJ), *O. ridleyi* (HHJJ) and *O. schlechteri* (HHKK) (Qian and Chen 2006). So, establishing gene introgression through allopolyploid as a bridge role can

Table 5 Agronomic traits of diploid and amphiploid plants

Line or hybrid	Plant height (cm)	Spike length (cm)	Grain length/width (cm)	Awn length (cm)	Flag leave angle		Leaf area (cm^2)	Seed set (%)	Shattering trait
					≤30°	>30°			
Sgdts96-2x	62.70	16.73	0.68/0.25	0-0.04	100 %	0	33.14	88.14	Difficult
HN2026-2x	61.63	20.90	0.70/0.32	0-0.60	100 %	0	65.92	85.71	Difficult
Nipponbare-2x	79.30	20.70	0.65/0.34	0	100 %	0	32.67	81.30	Difficult
O. punctata-2x	55.87	15.94	0.65/0.25	2.10-5.5	4.63 %	95.47 %	15.64	12.32	Easy
CW008-2x	85.28	8.89	0.53/0.25	0.22-2.81	14.29 %	85.71 %	15.26	0	Easy
DCW008-4x F_1	114.80	18.31	0.84/0.29	0.10-4.72	55.93 %	44.07 %	20.07	31.52	Easy
DCW008-4x F_2	115.21	20.90	0.82/0.31	0.34-5.10	50.12 %	49.88 %	26.21	37.65	Easy
DCW008-4x F_3	115.82	18.74	0.81/0.33	0.31-4.50	53.64 %	46.36 %	25.37	35.65	Easy

improve efficiency to transfer useful genes from the wild species. In the present study, chromosome fragment introgression and chromosome recombination could be observed through GISH investigations which were performed on synthetic amphiploid DCW008 (Fig. 1d). Multani et al. (1994) produced a triploid hybrid by crossing an artificial tetraploid of *O. sativa* and normal diploid *O. australiensis* and developed monosomic alien addition lines and gene introgression. Mariam et al. (1996) doubled interspecific hybrid F_1 and backcrossed with cultivated rice. Finally, they obtained bacterial blight resistance from *O. minuta.*

Allopolyploid play an important role in breeding programs and the.genetic and evolutionary studies of wheat (Goncharov et al. 2007), cotton (Flagel et al. 2008), rape (Albertin et al. 2006) and so on. From an evolutionary point of view, a different genomic combination and polyploidization reflect the general direction of the evolution of crops (Cai et al. 2001). Synthetic allopolyploid rice will probably produce new polyploid germplasm resources just as wheat, cotton, rape and groundnut once did.

Homoeology of the A and B genome

There are many studies involved relationship between A and B genome, usually amongst A, B and C. Qiu et al. (2010) considered that B and C genomes had genetic relationship with A which was the basic genome. Through the phylogenetic tree of the genus *Oryza*, genetic relationship between A and B is closer than A and C (Nishikawa et al. 2005). Gao et al. (2007) had the same viewpoint. In this investigation, meiotic chromosome pairing in diploid F_1 hybrids of *O. sativa* and O. *punctata* was irregular, with mostly univalents. But 73.42 % of the pollen mother cells (PMCs) had means 4.89–8.52 bivalents. The meiotic prophase I of the PMCs from the 31.31 % synthetic amphiploid hybrids had means 2.29 quadrivalents. 11.11 % of PMCs had more than four quadrivalents. Based on the pairing in interspecific diploid and amphiploid hybrids, it can be considered due to homoeologous pairings of chromosomes among A and B genomes. So, the occurrence of bivalents in diploid and quadrivalents in amphiploid could be attributed to allosyndesis. It indicated that the A and B genomes were partial homology, which is beneficial to transfer some useful genes from wild rice to cultivated rice.

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