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Cytomorphological Characterization of the Backcross Progeny of Synthetic Amphiploid Rice (AABB) and Tetraploid *Oryza sativa* (AAAA)

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Abstract

DCW008, a synthetic allopolyploid rice (2n = 4X = 48, AABB), was obtained from chromosome doubling of interspecific hybrids between *Oryza sativa* (2n = 24, AA) and *O. punctata* (2n = 24, BB). The F_1 and backcross (BC_1) hybrids were produced by crossing DCW008 as a female parent with a high seed set tetraploid rice Sg99012 - 4X (2n = 4X = 48, AAAA). BC_1F_1 and BC_1F_2 overcame many of the wild-type traits of DCW008; they had yellow-hulled grains, seed set ranged from 0% to 71.31% and the grain morphology was similar to that of cultivated rice. Variable numbers of chromosomes were observed in pollen mother cells (PMCs) from the BC_1 plant. Genomic *in situ* hybridization (GISH) revealed that the majority of somatic cells and PMCs contained six chromosomes of *O. punctata* with fragment recombination observed in two of them. The backcross selection method employed in this study to generate allopolyploid progeny provides a reliable way of transferring useful genes from wild species into cultivated rice.

Keywords: *Oryza punctate*, backcrosses, synthetic allopolyploid rice, genomic *in situ* hybridization (GISH), introgression

1. Introduction

The genus *Oryza* is composed of more than 20 species, represented cytogenetically by ten genome groups, including two cultivated species, *O. sativa* L. and *O. glaberrima* Steud (Li et al., 2001; Bao et al., 2006). Abundant wild species of *Oryza* provide extremely valuable genetic resources to broaden the genetic background of cultivated rice (Khush, 1977). Many researchers have, for a long time, tried to use these wild resources but reproductive barriers, especially at the diploid level, make it difficult to cross and transfer useful genes from wild rice to cultivated rice. However, allopolyploids, which have multiple chromosome sets, exhibit high plasticity since they have the evolutionary advantage of possessing additional genetic materials for growth and adaptation. Hence, they may play an important role in the distant hybridization and promote the exploitation of useful genes from wild species. Allopolyploidy has proven very useful in the utilization of genetic resources to increase yields in many crop species (Albertin et al., 2006; Goncharov et al., 2007; Flagel et al., 2008). Furthermore, allopolyploid rice is a versatile material that can be used to study relationships between different genome groups or in research on rice evolution. It can also be used as a bridge for transferring desirable genes into cultivated rice through gene introgression (Cai et al., 2001). This study utilized the synthetic allopolyploid rice DCW008 (AABB) (created by our laboratory and reported by Wang et al. 2013), which exhibits good fertility and advantageous agronomic traits. DCW008 was crossed and backcrossed with the high seed set tetraploid rice Sg99012-4X (AAAA) as a male parent to obtain the backcross derivatives. The genomic components and cytological characteristics of the BC_1 hybrids were studied using GISH. Grain yield and other major agronomic traits of F_1, BC_1F_1 and BC_1F_2 were also investigated.

2. Materials and Methods

2.1 Plant Materials

The synthetic amphiploid rice DCW008 (AABB, 2n = 4X = 48) was developed from chromosome doubling of interspecific hybrids between Sgdts96 (*Oryza sativa*, 2n = 24, AA) and *O. punctata* (2n = 24, BB). The cultivated rice Sg99012 (*O. sativa*, AA, 2n = 2X = 24) is a japonica rice line stored at the Polyploid Genetics Lab

of Hubei University, Wuhan, China. Sg99012-4X (AAAA, 2n = 4X = 48) is a high seed set tetraploid rice line with a polyploid meiosis stability (PMeS) gene selected by Cai et al. (2007).

2.2 Crosses and Backcrosses

DCW008 was used as the female parent in crosses with Sg99012-4X. Hybrid plants were generated through embryo rescue according to the procedure described by Wang et al. (2013). First backcross generations (BC_1) were generated using Sg99012-4X as a recurrent male parent. The progeny were also produced through embryo rescue. The method for the generation of materials is shown in Figure 1.

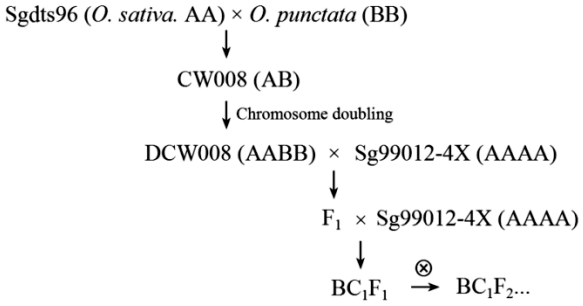

Figure 1. Scheme for production of backcross

2.3 Cytological Observations and GISH

Root tips from F_1 and backcross progeny were used for the determination of chromosome numbers. Directly fixed young inflorescences were used for meiotic behavior observation (Li et al., 1995). Pollen fertility was determined as the percentage of pollen grains stained with 0.2% fluorescein diacetate (FDA).

For GISH, root tip cells and selected anthers with pollen mother cells (PMCs) at suitable stages were digested for around 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 was extracted from young leaves using the hexadecyltrimethylammonium bromide (CTAB) method. The DNA of *O. punctata* was fluorescently labeled with bio-11-dUTP using nick translation and used as the probe. The genomic DNA from Sg99012-4X 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).

2.4 Morphological Observations

The key morphological traits of plant height, spike number and length, grain length and width, awn length, shattering trait, seed color and seed set were investigated. Recording methods and standards were set according to the protocols of Gai (1996).

3. Results

3.1 F_1 Hybrids and Backcross Progeny

Seed set in the DCW008 × Sg99012-4X cross was 11.73% and the germination of rescued embryos was 61.90%. Twelve F_1 hybrid plants were obtained in total. The seed set upon backcrossing the F_1 hybrids with the recurrent female parent ranged from 4.38% to 6.25% across different years. A total of 31 BC_1F_1 embryos were rescued, but only 10 germinated with a mean germination frequency of 34.10%. Six BC_1F_1 plants survived in total (Table 1).

Table1. Seed set and number of plants obtained in the synthetic allopolyploid rice and Sg99012-4X cross, and backcross progeny through embryo rescue

Generation	Spikelets pollinated (No.)	Seed set (%)		Embryos cultured (No.)	Germination		Plants obtained (No.)
		(No.)	(%)		(No.)	(%)	
F_1	179	21	11.73	21	13	61.90	12
$BC1F_1$	112*	7*	6.25*	7	3	42.86	1
	297**	13**	4.38**	13	3	23.08	3
	206***	11***	5.33***	11	4	36.36	2

Note: *Recorded in Hainan, China, April, 2011; **Recorded in Wuhan, China, August, 2012; *** Recorded in Wuhan, China, April, 2012. All embryos were rescued in Hubei University, Wuhan, China.

3.2 Morphology of F_1 Plants and Backcross Progeny

The majority of the F_1 plants exhibited matroclinous morphology. All were of tall stature and tillering (Figure 2A). They had grain-shattering traits, long red awns, purple stigma and black hulled-grains. F_1 plants had no seed set after selfing although immature embryos could occasionally be observed. Compared with F_1 plants, the BC_1 plants' morphology was more similar to that of Sg99012-4X. FDA staining indicated most of the pollen was fertile (Figure 2B). Anthers were normal and full (Figure 2C) and the seed setting rate of the BC_1F_1 plants ranged from 6.90% to 21.33% (Table 2). The shattering traits of BC_1F_1 were normal and the grain hull was yellow (Figure 2D, d1). Segregation occurred amongst the BC_1F_2 plants. Using grain hull color as an example, four plants (plant Nos. 6, 8, 12 and 19) produced black-hulled grains, but the remaining progeny had yellow-hulled grains. The grain-shattering trait also segregated into the normal and shattering phenotypes. The BC_1F_2 progeny had differing levels of seed set after selfing; six individuals (25%) had less than 5% seed set whereas the remaining plants (75%) had a seed set greater than 5%. Plant Nos. 3 and 7 had very high seed sets (71.31% and 61.32%, respectively).

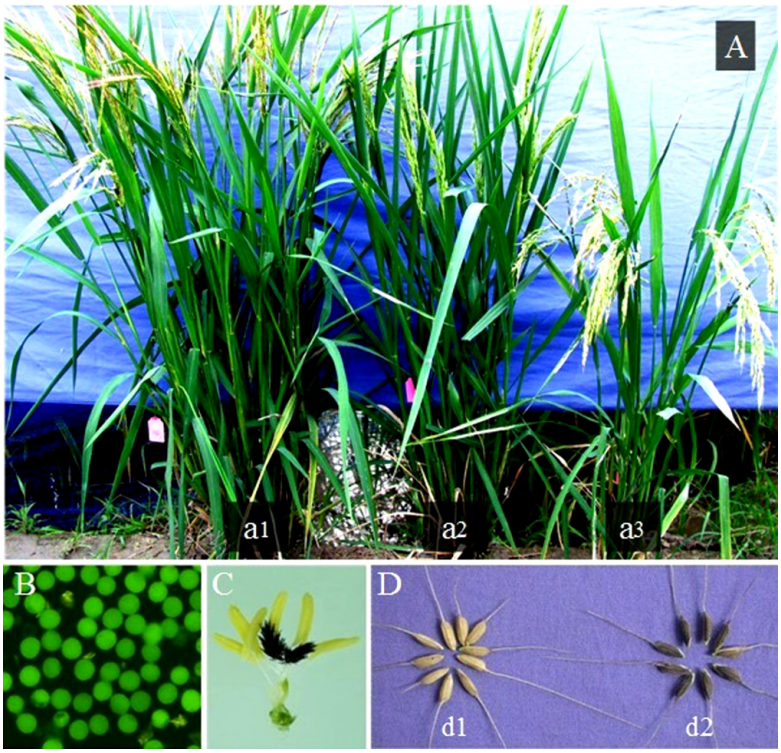

Figure 2. (A) Overall morphology of the hybrids. a1: F_1; a2: BC_1F_1; a3: Sg99012-4x. (B) Pollen staining by FDA. (C) Glumous flower of BC_1F_1 (no outer glume). (D) Seeds of BC_1F_1 and allopolyploid rice (AABB). d1: yellow, seeds of BC_1F_1; d2: black, seeds of AABB

Table 2. Agronomic traits of F_1, BC_1F_1 and BC_1F_2 from synthetic allopolyploid rice × Sg99012-4X

Materials		Plant height (cm)	Spike number	Spike length (cm)	Grain length / width (cm)	Awn length (cm)	Seed set (%)	Seed color	Shattering trait
Sg99012-4x		83.79	8	26.13	1.00 / 0.31	0 - 2.58	86.11	yellow	Normal
F_1		109.22	17	28.60	0.90 / 0.35	0.31 - 8.05	0	black	Shattering
BC_1F_1	Plant 1*	96.20	12	25.30	1.00 / 0.30	0.11 - 2.80	9.95	yellow	Normal
	plant 2**	79.62	13	21.18	1.00 / 0.29	0.23 - 3.09	15.92	yellow	Normal
	plant 3**	82.33	9	19.89	1.05 / 0.30	0.32 - 6.75	21.33	yellow	Normal
	plant 4**	83.97	10	21.36	1.00 / 0.29	0.44 - 5.28	12.74	yellow	Normal
	plant 5***	94.30	18	22.44	0.95 / 0.33	0.17 - 3.51	6.90	yellow	Normal
	plant 6***	105.19	14	29.72	1.00 / 0.33	0.06 - 2.96	18.37	yellow	Normal
BC_1F_2	Plant 1	116.11	15	33.11	0.85 / 0.31	0 - 2.60	18.37	yellow	Normal
	plant 2	108.55	13	30.15	1.10 / 0.30	0.61 - 2.20	32.67	yellow	Normal
	plant 3	93.37	15	25.23	1.00 / 0.30	0.30 - 2.70	71.31	yellow	Normal
	plant 4	85.30	12	25.77	1.00 / 0.35	0.10 - 1.61	33.82	yellow	Normal
	plant 5	78.60	9	28.42	0.95 / 0.35	0.51 - 2.62	28.89	yellow	Normal
	plant 6	70.67	16	26.30	1.00 / 0.35	1.62 - 4.70	17.46	black	Shattering
	plant 7	80.50	15	23.50	1.10 / 0.35	1.20 - 6.94	61.32	yellow	Normal
	plant 8	95.25	13	14.70	0.90 / 0.38	0.10 - 0.90	2.63	black	Shattering
	plant 9	103.36	11	25.89	1.00 / 0.35	0.33 - 6.10	46.81	yellow	Normal
	plant 10	92.60	16	23.95	0.95 / 0.33	0.10 - 3.21	20.24	yellow	Normal
	plant 11	90.50	9	24.45	1.00 / 0.35	0.66 - 4.60	25.51	yellow	Normal
	plant 12	79.8	14	23.90	1.00 / 0.35	0 - 3.15	0	black	Shattering
	plant 13	86.47	11	21.84	1.00 / 0.35	0 - 2.13	3.85	yellow	Normal
	plant 14	110.96	9	27.40	0.91 / 0.38	0.31 - 4.90	3.16	yellow	Normal
	plant 15	74.33	13	19.26	0.92 / 0.38	0.11 - 3.40	38.53	yellow	Normal
	plant 16	95.20	17	26.19	0.98 / 0.36	0 - 1.10	2.91	yellow	Shattering
	plant 17	106.69	13	22.10	0.98 / 0.35	0 - 0.61	36.52	yellow	Normal
	plant 18	102.53	18	25.40	1.00 / 0.40	0 - 1.00	41.13	yellow	Normal
	plant 19	86.30	15	23.80	1.00 / 0.35	0.21 - 1.75	5.85	black	Shattering
	plant 20	99.60	11	25.35	1.00 / 0.35	1.20 - 6.65	19.26	yellow	Normal
	plant 21	79.05	9	20.00	0.95 / 0.36	0.60 - 2.80	0	yellow	Normal
	plant 22	92.90	12	21.30	0.90 / 0.38	0.34 - 4.50	29.75	yellow	Shattering
	plant 23	88.26	12	22.27	0.90 / 0.38	0 - 0.92	10.62	yellow	Normal
	plant 24	86.54	15	23.80	1.00 / 0.38	0 - 0.41	22.14	yellow	Normal

Note: *Recorded in Wuhan, China, 2011; **Recorded in Hainan, China, 2012; *** Recorded in Wuhan, China, 2012. The BC_1F_2 plants were the progeny of plant 1 of BC_1F_1.

3.3 Meiotic Behavior of the BC_1F_1 Plant

The BC_1F_1 plant generally showed irregular meiosis. Univalent, bivalent, trivalent, quadrivalent and multivalent chromosomes could be observed (Figure 3, b-d). There were mainly four types of chromosome pairing at diakinesis/ metaphase I (Table 3). Lagging chromosomes were observed frequently in PMCs at metaphase I and

anaphase I (Figure 3, e-f). Four lagging chromosomes were observed most frequently (42.31%) with one lagging chromosome seen the most infrequently (3.85%; Table 4). Chromosomal bridges were also observed in some PMCs at anaphase I (Figure 3, g).

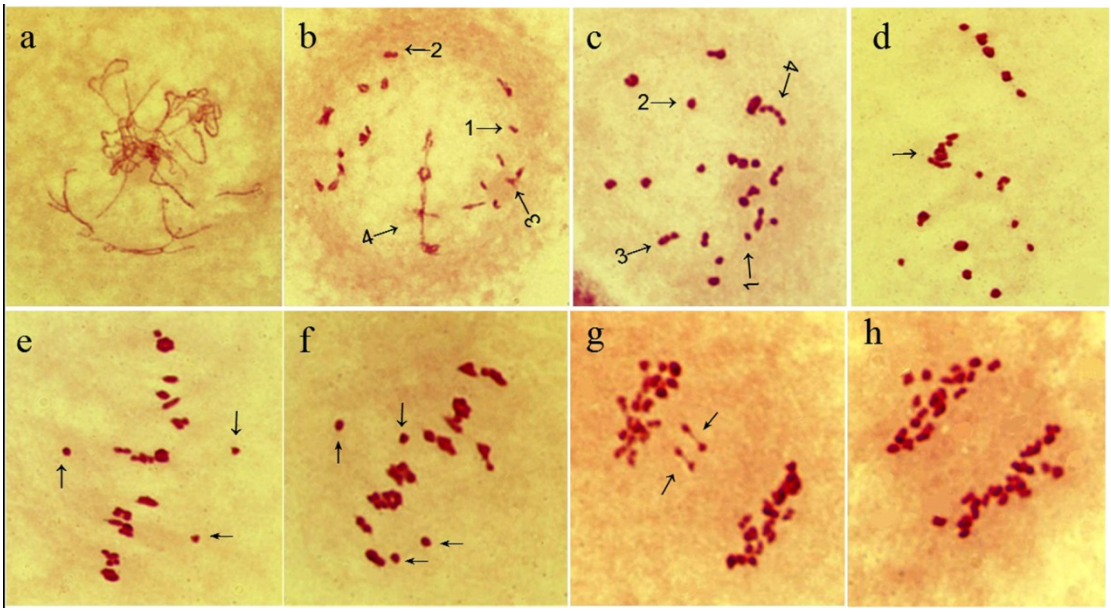

Figure 3. Meiotic aspects of the $BC_1F_1$from synthetic allopolyploid rice (AABB) × Sg99012-4X (*O. sativa*, AAAA, 2n = 4X = 48). (a) zygotene (prophase I); (b)chromosomes at diplotene (prophase I), arrow 1: univalent; arrow 2: bivalent; arrow 3: quadrivalent; arrow 4: multivalent, c-f: Chromosomes at diakinesis (prophase I), (c) arrow 1: univalent; arrow 2: bivalent; arrow 3: trivalent; arrow 4: quadrivalent, (d) arrow: multivalent. e-f: Two PMCs at metaphase I showing lagging chromosomes (arrows). g-h: Two anther PMCs at anaphase I. (g) with two chromosome bridges; arrows. (h) normal

Table 3. Chromosome pairing of the backcross-1 plant having 2n = 48

Types	Number of PMCs	Chromosome pairing	Percentage (%)
Type 1	27	4.0 Ⅰ + 7.69 Ⅱ + 7.15Ⅳ	27.84
Type 2	36	3.72 Ⅰ + 6.35 Ⅱ + 3.62Ⅲ + 5.18Ⅳ	37.11
Type 3	23	4.17 Ⅰ + 1.42 Ⅱ + 1.67Ⅲ + 5.50Ⅳ + 2.33 multivalent	23.71
others	11		11.34

Note: I univalent, II bivalent, Ⅲ trivalent IV, quadrivalent.

Table 4. Lagging chromosomes of the meiotic metaphase I and anaphase I of the backcross-1 plant

Number of lagging chromosomes	Number of PMCs	Percentage (%)
1	5	3.85
2	14	10.77
3	24	18.46
4	55	42.31
5	32	24.62

3.4 GISH Analysis of the BC_1F_1 Plant

The chromosome number in root tips of the BCF_1 plant was 2n = 48. GISH analysis indicated that four chromosomes were wholly labeled by the *O. punctata* probe. Two chromosomes were partly labeled (Figure 4,

a1-2). In some diakinesis PMCs four univalents were fully labeled and two bivalents were partly labeled but the attached fragments were unlabeled (Figure 4, b1-2). At metaphase I of some PMCs, two partly labeled bivalents appeared in the equatorial plate, whilst four lagging chromosomes were labeled. In addition, there was another unlabeled lagging chromosome (Figure 4, c1-2).

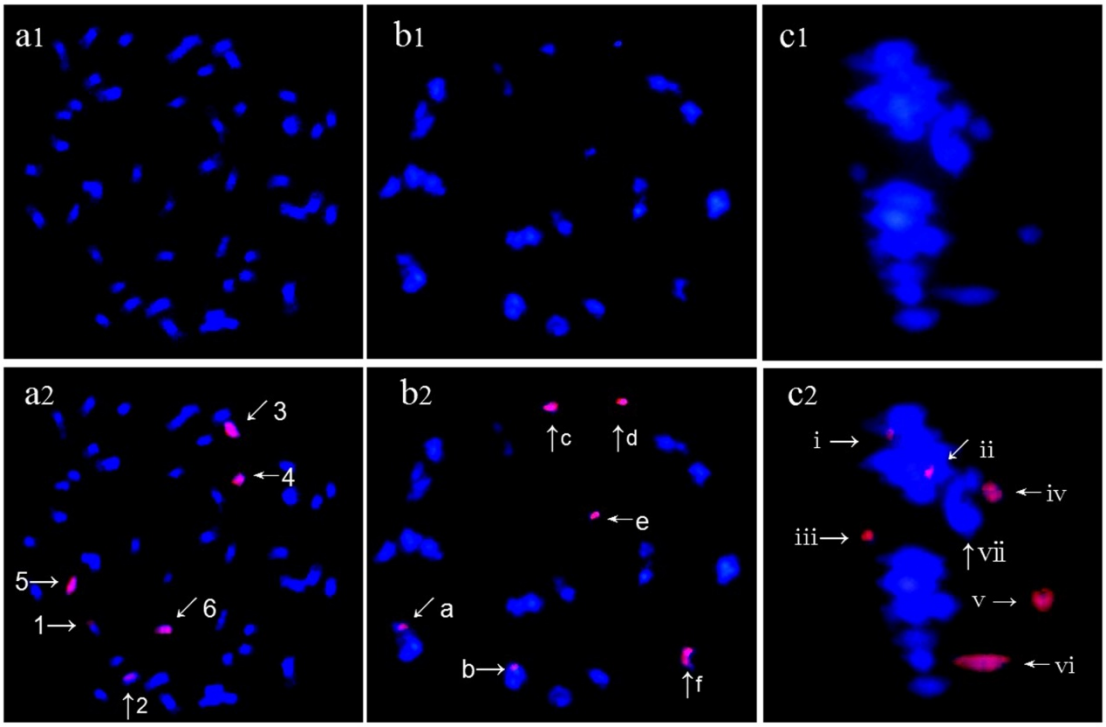

Figure 4. GISH analysis. DAPI (blue) and merged (red signals from the *O. punctata*) images. a1 - a2: Chromosomes of somatic cell from BC_1F_1 (2n = 4X = 48). (a1) DAPI image; (a2) Two chromosomes (arrows 1, 2) were partly labeled. 4 chromosomes were fully labeled (arrows 3-5). b1 - b2: one PMC at diakinesis. (b1) DAPI image; (b2) Two bivalent (arrows a, b) were labeled but the attached fragments were unlabeled. The univalents (arrows c-f) were fully labeled. c1 - c2: one PMC at metaphase I. (c1) DAPI image. (c2) Two partly labeled bivalent arranged in equatorial plate (arrows ⅰ, ⅱ). Four Lagging chromosomes were fully labeled (arrows ⅲ-ⅵ). There was another unlabeled laggard (arrow ⅶ)

4. Discussion

4.1 Utilization of Desirable Genes from Wild Rice Resources

The wild *Oryza* species are an important source of desirable genes for resistance to major pests and diseases and for tolerance to various abiotic stresses. As such they form an extremely valuable genetic resource (Flagel et al., 2008; Multani et al., 2003). Researchers have attempted to use these wild resources for many decades. During the 1970's several interspecific hybrids and amphiploids were produced in order to introgress novel genes from wild species into cultivated rice (Khush, 1977). Many disease and insect resistance genes introgressed from wild species have been used in rice breeding and several varieties carrying these genes have now been released (Jena and Khush, 1989; Amante-Bordeos et al., 1992; Multani et al., 1994). Some wild species genes (*Xa 21, Bph 18*) have also been used in marker assisted selection (Singh et al., 2001; Jena et al., 2006). The purpose of this study was to obtain new rice germplasm and to introgress genes from *O. punctata* on a tetraploid level. In this investigation, the F_1 plants were sterile. However, improvements in three agronomically important traits were observed in the BC_1F_1 and BC_1F_2 plants- increased seed setting rate, normal shattering trait and short plant height. New polyploid rice lines (2n = 48), monosomic alien addition lines or gene introgression lines with *O. punctata* chromosome fragments or genes will now be obtained from this backcross progeny.

4.2 Effects of allopolyploidy in Crop Breeding

Polyploidy is an evolutionary innovation in many plant and some animal species (Ni et al., 2009). Allopolyploid plants are hybrids that contain two copies of the genome from each parent (Comai, 2000). Many commercially important crops such as wheat, cotton and canola are allopolyploids (A. R. Leitch, & I. J. Leitch, 2008; Chen, 2007). In addition, some allopolyploid rice species already exist in the wild. Synthetic allopolyploids should further enrich existing rice resources. Moreover, they will be beneficial for both the protection of wild resources and the storage and use of wild germplasms (Sangiacomo & Sullivian 1994; Kazi et al., 1996; Ge & Li, 2007; Goncharov et al., 2007; Yao et al., 2012). From an evolutionary perspective, different genomic combinations and polyploidization reflects the general direction of crop evolution (Cai et al., 2001). Allopolyploids possess the evolutionary advantage of having double the genetic material for growth and adaptation and can overcome the reproductive barrier of wild and cultivated species (Wang et al., 2013). Hence, they may play an important role in the distant hybridization and assist plant breeders in the utilization of genetic resources from wild species.

5. Conclusions

Obtaining desirable genes from wild species is an important approach in plant breeding. In combination with wild cross, polyploidization and backcrossing, the polyploids or allopolyploids will assist plant breeders in the utilization of genetic resources from wild species.

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