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Development and identification of synthetic interspecific hybrids between *Oryza sativa* and *Oryza australiensis*

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Abstract

***Oryza sativa* and *Oryza australiensis* are both diploid and containing AA and EE genomes, respectively. Although their distant genetic relationship makes crossing difficult, interspecific hybrids between the two species were obtained by young embryo rescue in this study. The F_1 hybrids resembled the wild rice parent, *O. australiensis*, in respect of morphological traits such as plant height, lengths and widths of flags and second leaves, panicle type and length, long-awned spikelets, grain shattering, sensitivity to photoperiod and tillering capability. The genomic components and chromosome pairing of the hybrids were subsequently investigated using a genomic *in situ* hybridization analysis. Based on the mitotic metaphase chromosomal numbers in the root tips, all these hybrids are characterized to be allodiploids with 24 chromosomes, and their genomic constitution is AE. A poor chromosomal pairing was identified in meiotic anaphase I of the hybrid pollen mother cell as most of the chromosomes were univalent. This work laid the foundation for transferring favorable traits and useful genes from *O. australiensis* to cultivated rice in the near future.**

Key words: *Oryza australiensis*, embryo rescue, interspecific hybrid, genomic *in situ* hybridization, allodiploid

Introduction

Rice (*Oryza sativa*) is the most important human food source for more than a third of the world's population. During the last few decades, major advances have been made in increasing rice yields, but rice productivity is continually threatened by several biotic and abiotic stresses. The genetic variability in response to these stresses is limited in the cultivated rice germplasm. Thus, there is an urgent need to broaden the rice gene pool by transferring genes for such traits from diverse sources (Brar and Khush 1997). Wild rice species are grown worldwide under a range of complex agroclimatic conditions and offer a largely untapped resource of agriculturally important genes that have the potential to solve many problems associated with rice production (Wing et al. 2005). Hybridization between *O. sativa* (AA genome) and its wild species is an important and acceptable plant-breeding method to broaden the genetic base and transfer useful genes in to cultivated rice. Several useful genes from wild species have been successfully transferred, including the genes for resistance to grassy stunt virus from *O. nivara*, for bacterial blight from *O. longistaminata*, and cytoplasmic male sterility from *O. rufipogon* (Brar and Khush 1997). These wild species that share the AA genome with *O. sativa* can be easily crossed and useful genes may be transferred through conventional hybridization procedures.

In addition to the two cultivated rice species (*O. sativa* and *O. glaberrima*) and six wild species having the AA genome, the genus *Oryza* contains 16 wild species with genomes other than AA (non-AA), *viz.,* BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ and KKLL (Vaughan et al. 2003; Lu et al. 2009). The genomic differences between the non-AA and AA genomes make it difficult to produce interspecific hybrids. With the development of the embryo culturing technique, some interspecific hybrids were obtained, and a few useful genes from non-AA genome containing wild

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species were transferred and identified, including genes for resistance to brown plant hopper from *O. officinalis* (Wang et al. 2001) and *O. eichingeri* (Liu et al. 2001), bacterial blight and blast resistance from *O. minuta* (Amante-Bordeos et al. 1992) and whitebacked plant hopper resistance from *O. officinalis* (Tan et al. 2004). However, the information on the use of *O. australiensis* is limited. To efficiently enhance germplasm exploitation of *O. australiensis*, it is necessary to obtain interspecific hybrids between *O. sativa* and *O. australiensis*. In the present study, an attempt was made to produce interspecific hybrids between these species adopting embryo rescue technique. Subsequently, the hybrids were investigated using morphological and molecular cytogenetic methods.

Materials and methods

Plant materials

Three *japonica* cultivars, Nipponbare, Haifeng 1 and Zhonghua 11 of *O. sativa* (2n = 2X = 24, AA genome), and two accessions (Acc.) of *O. australiensis* (2n = 2X = 24, EE genome, Acc.105278 and 103318) were used in this experiment. To ensure the same timing for heading under the ecological conditions found in Yangzhou, China, *O. australiensis* and interspecific hybrids were exposed to a short day treatment (8 h of bright sunlight per day). The treatment started at the peak of tillering and continued more than 20 days(d).

Hybridization, embryo rescue, somatic and meiotic preparations

The spikelets of the *japonica* varieties were emasculated by dipping in water at 44-45°C for 5 min. before anthesis and were subsequently pollinated by *O. australiensis*. The pollinated spikelets were wrapped in Kraft paper bags and sprayed with a solution of 75 ppm gibberellin 3 d after pollination (Jena and Khush 1984). Embryos rescue was conducted as described previously (Yi et al. 2008). Fresh root tips were harvested from interspecific hybrids grown in the field and pretreated in 0.0002 M 8-hydroxyquinoline at 20°C for 2 h, then fixed in methanol:acetic acid (3:1) and stored at –20^{o}C until use. The root tips were macerated in 2% cellulose and 1% pectinase at 37^{o}C for 1 h, then squashed and flame-dried on glass slides. Young panicles of the hybrids were collected and fixed in 3:1 (100% ethanol:glacial acetic acid) Cannoy's solution. Microsporocytes at the anaphase I stage were squashed in acetocarmine solution, and the slides were flame-dried.

Genomic in situ hybridization (GISH) analyses

At the tillering stage, 5 g fresh young leaves of *O. australiensis* were collected and extracted the total genomic DNA using the sodium dodecyl sulfate method (Kang and Yang 2004), then labeled as the probe with digoxigenin-11-dUTP (Boehringer Mannheim Biochemical) using the nick translation method. GISH analyses were conducted according to the protocol of Cheng et al. (2002). Somatic mitotic chromosomes and meiotic anaphase I chromosomes were hybridized with the above digoxigenin-labeled probes. The chromosomes were counterstained with 4',6-diamidine-2-phenylindole dihydrodihydrochloride (DAPI) in an antifade solution (Vectashield; Vector Laboratories, Burlingame, CA, USA). Images were captured using a chilled charge-coupled device (CCD) camera mounted on an Olympus BX61 fluorescence microscope and merged using IPLab software.

Results

Production of interspecific hybrids

A total of 44 F_1 hybrid seeds were obtained after pollinating 459 spikelets from the three cross combinations (Table 1). The seed setting percentages ranged from 6.70-13.10%, depending upon the rice variety used. Seed set from the cross of Haifeng 1 × *O. australiensis* Acc.105278 was 13.10%, which was the highest, followed by the seed set in the cross,

Table 1. Fertilities of interspecific crosses between *O. sativa* and *O. australiensis*

Crosses	No. of spikelets pollinated	No. of embryos cultured	Seed set (%)	No. of embryo germi-nation	Embryo germi-nation (%)	No. of hybrids	Crossability (%)
Nipponbare/*O.australiensis* (Acc.105278)	90	10	11.11	3	30.00	2	2.22
Haifeng 1/*O.australiensis* (Acc.105278)	145	19	13.10	5	26.32	3	2.07
Zhonghua 11/ *O.australiensis* (Acc.103318)	224	15	6.70	4	26.67	2	0.89

Nipponbare × *O. australiensis* Acc.105278 (11.11%) and Zhonghua 11 × *O. australiensis* Acc.103318 (6.70%). In Nipponbare × *O. australiensis* Acc.105278 cross, 3 of the 10 immature hybrid embryos cultured on the medium germinated. Two plantlets were eventually obtained. The crossability was 2.22%, which was the highest of the three cross combinations. Under the same culture conditions, the crossability of the Zhonghua 11 × *O. australiensis* Acc.103318 cross was the lowest.

Morphological features of interspecific hybrids

The hybrids from the cross of Nipponbare × *O. australiensis* Acc.105278 were selected for the following study. As shown in Table 2, the F_1s resembled *O. australiensis* in growth and in some morphological features such as the plant height (Fig. 1A), lengths and widths of flag and second leaves, panicle type and length (Fig. 1B), shattering, sensitivity to photoperiod and tillering capability. The hybrids were uniform, male-sterile, with long-awned spikelets and well-exerted panicles (Figs. 1A and 1C). The potassium iodide (1% I2-KI) staining, revealed the normal darkly stained pollen grains of the two parents (Figs. 1D1 and 1D2), but those of the hybrids were typically and spherically abortive (Fig. 1D3). Based on the plant phenotypes, the hybrids were true interspecific hybrids.

Table 2. Comparison of morphological traits among *O. sativa* × *O. australiensis* F_1 hybrids and their parents

Traits	Nipponbare/*O. australiensis* Acc. 105278		
	Nippon-bare	*O. austra-liensis*	F_1
Plant height (cm)	108.33± 2.08 *	160.50± 2.12	122.67± 1.53
Length of flag leaf (cm)	40.33± 2.31	31.60± 1.13	32.30± 2.19
Width of flag leaf (cm)	1.43± 0.06	1.85± 0.07	1.93± 0.11
Length of second leaf (cm)	47.67± 4.51	50.63± 3.61	50.65± 3.24
Width of second leaf (cm)	1.20± 0.00	1.75± 0.07	1.77± 0.06
Length of panicle (cm)	19.70± 0.52	33.10± 0.57	20.10± 0.65
Grain number per panicle	97.67± 3.79	221.00± 4.24	115.67± 3.06
Panicle type	compact	open	open
Shattering	weak	strong	strong
Sensitivity to photo-period	weak	strong	strong
Tillering capability	intermediate	weak	weak

*all data were obtained from 5 plants and shown by Mean±SD

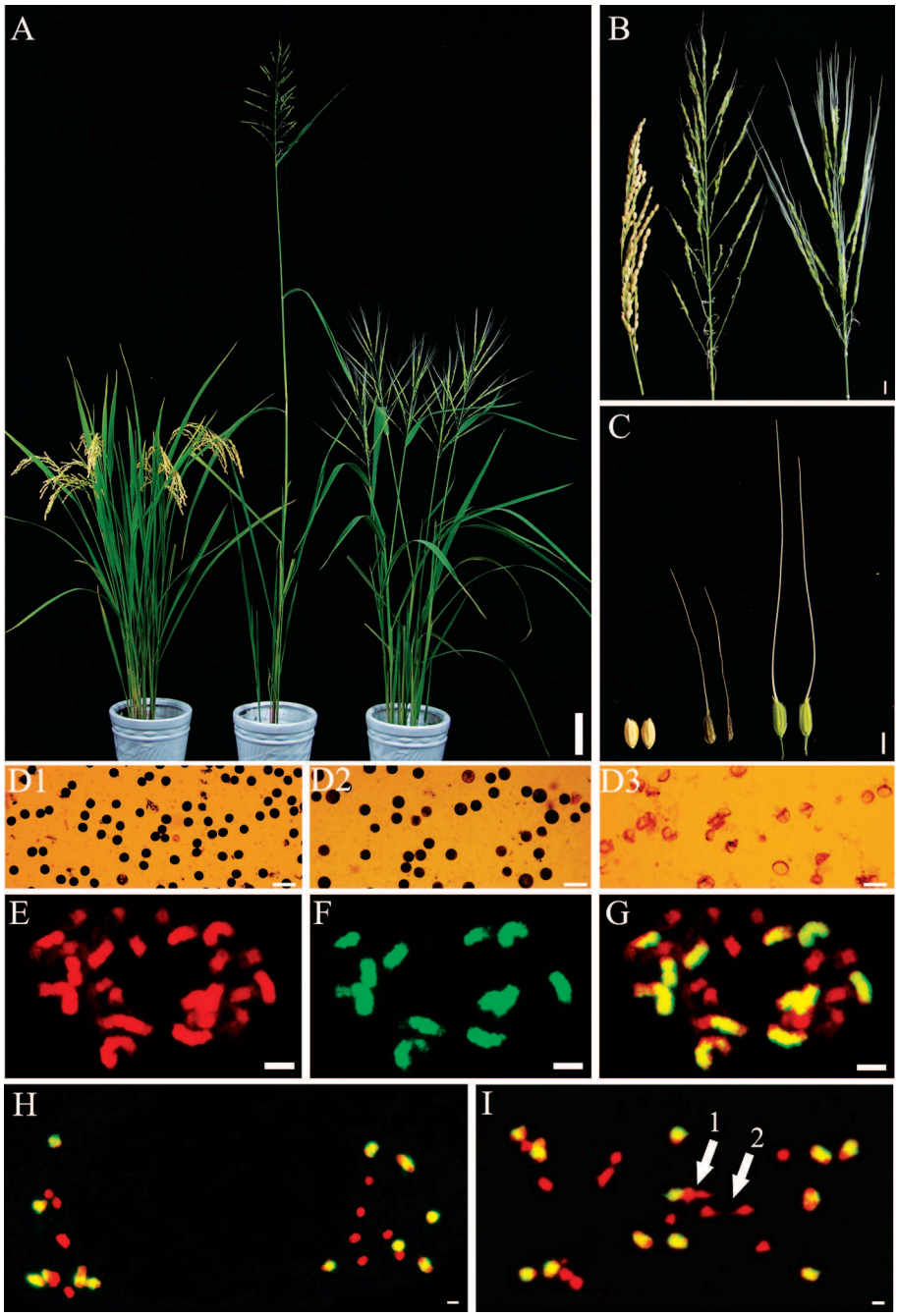
GISH analysis of interspecific hybrids

The GISH analysis showed that each root-tip cell of the interspecific hybrids had 24 chromosomes (Fig. 1E), the 12 green chromosomes (Fig. 1F) or 12 greenish-yellow chromosomes (Fig. 1G) originated from the male parent, *O. australiensis*, while the 12 red chromosomes (Fig. 1G) originated from the female parent, *O. sativa*. Thus, there were two genomes in every somatic cell of the hybrids. Combined with the genome construction of the parents, it was deduced that the interspecific hybrids were allodiploid and had AE genomic constitution. To analyze further, the chromosomal pairing of the hybrid, the same GISH program was conducted on the meiotic anaphase I chromosomes of the hybrid pollen mother cells. Chromosomal pairing of the hybrid was distinguished and investigated in 25 cells. As depicted in Table 3, a majority of the chromosomes were univalents, with 6 of 25 investigated pollen mother cells all showing 24 univalents (Fig. 1H). The average number of univalent chromosomes was 22.08 (range 18-24) per cell. On average, the A and E genomes of the hybrid had 10.80 and 11.28 univalent chromosomes in each cell, respectively. A low frequency of bivalents was recorded in the target cells. Arrows 1 and 2 in Fig. 1I indicate an A-E and A-A bivalent, respectively. The average number of bivalent chromosomes was 0.96

Table 3. Chromosome pairing of different genomes observed at meiotic anaphase I in the interspecific hybrid between *O. sativa* and *O. australiensis*

Manner of chromosome synapsis	Type	Mean±SD*	Range
Univalents	A	10.80±1.15	8-12
	E	11.28±0.79	10-12
Bivalents	A-A	0.36±0.00	0-1
	E-E	0.12±0.00	0-1
	A-E	0.48±0.42	0-2

*:Mean values of different chromosome associations observed at meiotic anaphase I in 25 hybrid pollen mother cells



(range 0-2) per cell. The average frequency and range of chromosome associations in the meiotic anaphase I pollen mother cells of the hybrid were 2n = 24 = 22.08 (18-24) I + 0.96 (0-2) II.

Discussion

In general, low crossability, which is a post-fertilization reproduction barrier, is a common phenomenon encountered in wide hybridization due to the great genomic differences between the parents (Chen et al. 2016). In the genus *Oryza*, interspecific hybridization, especially involving intergenomic crosses, is normally characterized by low crossability, which is commonly less than 10% (Mariam et al. 1996). However, with the development of embryo rescue techniques, some interspecific hybrids have been produced between *O. sativa* with the AA genome and distantly related wild species with non-AA genomes. The crossability of the interspecific hybrids was 0.36-1.62% between *O. sativa* and *O. eichingeri* (2n = 2X = 24, CC genome) (Yan et al. 1997), 1.55-5.30% between *O. sativa* and *Oryza alta* (2n

Fig. 1. Plant phenotypes and GISH analysis of interspecific hybrids between *O. sativa* and *O. australiensis.* A : plant phenotype, Scale bar: 10cm; B : Panicle morphology, Scale bar: 1cm; C : Grain morphology, Scale bars: 0.5cm; From left to right of all above pictures: *O. sativa* (Nipponbare), *O. australiensis* (Acc. 105278), F_1, respectively; D1-D3 : Pollen grains stained by potassium iodide (1% I2-KI); Scale bar: 50 μm; D1 = *O. sativa* (Nipponbare), D2 : *O. australiens* (Acc. 105278), D3: F_1; E : A complete set of somatic chromosomes of the interspecific hybrids were stained with DAPI (red), F : GISH signal derived from the genomic DNA probe of *O. australiensis* (green), G : A merge image of the chromosomes(E) and the GISH signals(F). Scale bars: 10 μm; H-I : GISH images of the meiotic anaphase I chromosomes of the hybrid probed by *O. australiensis* genomic DNA (greenish-yellow signals). The 12 greenish-yellow chromosomes in H and I are from *O. australiensis*, while the 12 red ones are from *O. sativa.* Chromosomes were counterstained with DAPI in all images and pseudocolored in red. Arrows point to bivalent chromosomes. 1, A-E bivalent; 2, A-A bivalent. Scale bars: 10 μm. Twenty-five hybrid pollen mother cells were observed

= 4X = 48, CCDD genome) (Fu et al. 2007) and 0-4.89% between *O. sativa* and *Oryza latifolia* (2n = 4X = 48, CCDD genome) (Yi et al. 2008). In the present study, the crossability of the hybrids between *O. sativa* and *O. australiensis* was 0.89-2.22% (Table 1). Obtaining interspecific hybrids is required for transferring useful genes from *O. australiensis* into cultivated rice. Backcross programme related to the present study is under progress.

GISH is a very effective method for investigating and identifying alien genomes and chromosomes (chromosome segments) in allopolyploids, wide hybrids, and their derivatives. Because genetic differences occur during long-term evolution, GISH has been widely used to study the differentiation and the resulting relationships in the genus *Oryza* (Li et al. 2001; Lan et al. 2006; Xiong et al. 2006). The A and E genomes involved in this study occurred genomic differentiation about 8.5 million years ago (Piegu et al. 2006), so it is easy to discriminate the A and E genomes of interspecific hybrids between *O. sativa* and *O. australiensis* (Fig. 1E-1I), and to investigate chromosome pairing at meiotic anaphase I in the pollen mother cells of the hybrid by the GISH procedure. The genomic differences between the A and E genomes of the hybrid might result in the chromosomes mostly existing as univalents (Fig. 1H-1I). But in the twenty-five investigated pollen mother cells, only one cell showed the univalents of the F_1 hybrid were equationally divided and migrated to the two poles in the anaphase I stage (Fig. 1H), which was maybe caused by meiotic restitution. We obtained two F_2 plants and five BC_1F_1 plants through a large number of selfing and backcross operations (data not shown). The F_2 plants were completely spikelet sterile, however the BC_1F_1 plants had different degrees of spikelet fertility ranged from 0.29%-91.02%. This study is helpful for developing chromosome introgression lines and transferring valuable genes from *O. australiensis* into cultivated species in the future research.

Authors' contribution

Conceptualization of research (CY, MG); Designing of the experiments (CY, YZ, GZ, MG); Contribution of experimental materials (CY, MG); Execution of field/ lab experiments and data collection (CY, DH, JZ, WJ, WL); Analysis of data and interpretation (DH, JZ, WJ); Preparation of manuscript (CY, DH, JZ, WJ).

Declaration

The authors declare no conflict of interest.

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