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Interspecific hybridization of cultivated rice, *Oryza sativa* L. with the wild rice, *O. minuta* Presl.

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Abstract Crosses were made between four varieties ('Mahsuri', 'Setanjung', 'MR84' and 'MR103') of *Oryza sativa* L. (2n = 24, AA) and one accession of *O. minuta* (2n = 48, BBCC). The seed set obtained ranged between 9.5% and 25.1% depending on the rice variety used. By rescuing 14-day-old embryos and culturing them on 25%-strength MS medium we obtained a total of 414 F_1 hybrids. The F_1s were vigorous, tillered profusely, were perennial and male-sterile. The hybrids were triploid (ABC) with 36 chromosomes and showed irregular meiosis. The average frequency and range of chromosome associations at metaphase I or early anaphase I pollen mother cells of F_1 plants were 29.31(16–36) Is + 3.32(0–10) IIs + 0.016(0–1) IIIs + 0.002(0–1) IVs. Upon backcrossing the original triploid hybrids and colchicine-treated hybrids to their respective recurrent parents, and further embryo rescue, 17 backcross-1 (BC_1) plants were obtained. Of all the crosses using MR84, no BC_1 plant was obtained even after pollinating 13 894 spikelets of the triploid hybrid. The BC_1s were similar in appearence to the F_1s and were male-sterile, their chromosome number ranged from 44 to 48. By backcrossing these BC_1s and nurturing them through embryo rescue, we obtained 32 BC_2 plants. Of these, however, only 18 plants grew vigorously. One of these plants has 24 chromosomes and the other 17 have chromosome numbers ranging between 30 and 37. The 24-chromosome plant was morphologically similar to the *O. sativa* parent and was partially fertile with a pollen and spikelet fertility of 58.8% and 12.5% respectively. All of the F_1 and BC_1 plants were found to be resistant to five Malaysian isolates (XO66, XO99, XO100, XO257 and XO319) of *Xanthomonas campestris* pv *oryzae*. Amongst the BC_2s, the reaction varied from resistant to moderately susceptible. The 24-chromosome BC_2 plant was resistant to the four isolates and moderately resistant to isolate XO100 to which the *O. sativa* parent was susceptible.

Key words *Oryza sativa* · *O. minuta* · Interspecific hybridization · Embryo rescue · Resistance to bacterial blight

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Introduction

The genus *Oryza* to which cultivated rice, *Oryza sativa* (2n = 24, AA genome) belongs, has more than 20 wild species. These wild species are a rich source of useful genes for the improvement of cultivated rice, most notably genes for resistance to insect pests (Heinrichs et al. 1985) and diseases (Sitch 1990). A number of useful genes have been transferred from wild species into cultivated rice; for example, genes for resistance to grassy stunt virus from *O. nivara* (Khush 1977) and for resistance to bacterial blight from *O. longistaminata* (Khush et al. 1990) have been transferred to *O. sativa*. These transfers involved closely related wild species with the AA genome that can be easily crossed with *O. sativa*.

The utilization of more distant wild species has become increasingly important in current rice breeding. According to Sitch and Romero (1990) these wild species with different genome(s) are the most valuable donors. However, gene transfer from these wild species to *O. sativa* proved to be extremely difficult. Success was always limited by low seed set, hybrid inviability, hybrid sterility and lack of chromosome recombination (Sitch et al. 1989a). With the refinement in tissue culture

techniques, particularly in the area of embryo rescue, some of the barriers have been overcome. Jena and Khush (1990) have successfully transferred genes for resistance to brown planthopper, Asia's most serious rice pest, and for whitebacked planthopper from *O. officinalis* to cultivated rice by hybridization, embryo rescue, backcrossing and selection. More recently, genes for resistance to blast, bacterial blight and brown planthopper have been transferred to cultivated rice from *O. minuta* (Amante-Bordeous et al. 1992), *O. latifolia* (IRRI 1993) and *O. australiensis* (Multani et al. 1994) by the same procedure.

Of particular interest is *O. minuta* J.S. Presl. ex C.B. Presl., an allotetraploid ($2n = 4x = 48$) species with a genomic composition of BBCC, which is distinct from *O. sativa*. This wild species, which is native to Asia, is resistant to rice blast, bacterial leaf blight, brown planthopper and whitebacked planthopper (Heinrichs et al. 1985).

This study was undertaken to explore the possibility of transferring gene(s) for resistance to bacterial blight from *O. minuta* to *O. sativa* by wide hybridization.

Material and methods

Materials

Four varieties of rice, *Oryza sativa*, 'Mahsuri', 'Setanjung' ('MR1'), 'MR84' and 'MR103', and one accession of wild species *O. minuta* (IRRI's Acc. No. 101141) were used in the study. All four rice varieties are modern improved varieties that were released in Malaysia in 1965, 1979, 1986 and 1990, respectively.

'Mahsuri' and 'Setanjung', are moderately resistant and resistant to bacterial blight, respectively (Othman et al. 1986); 'MR84' is susceptible to bacterial blight (Othman et al. 1994); 'MR103' is moderately resistant to bacterial blight (Chen et al. 1990). *O. minuta* is known to exhibit a high level of resistance against representative isolates of six Philippine *Xanthomonas campestris* pv. *oryzae* (Xco) races (Sitch et al. 1989b).

Five isolates of *X. campestris* pv. *oryzae* from Malaysia, namely XO66 (race 7), XO99 (race 5), XO100 (race 15), XO257 (race 1) and XO319 (race 7), were used to evaluate the resistance to bacterial blight.

All materials used in this study were kindly supplied by the Rice Division, Malaysian Agricultural Research Development Institute (MARDI), Kepala Batas, Seberang Prai. The plants were grown in the greenhouse at Universiti Kebangsaan Malaysia, Bangi.

Methods

Crosses and embryo culture

A number of crosses were made between *O. sativa* varieties as female parents and *O. minuta* as the male parent. For each variety, a total of 8 plants were pollinated. In backcrosses, the *O. sativa* varieties were used as the recurrent male parents. All of the F_1 hybrids obtained were backcrossed to their respective recurrent male parent to produce backcross-1 (BC_1) progenies. The BC_1 plants obtained were again backcrossed to their respective male parent to produce BC_2 progenies. Since the F_1 and BC_1 plants were male-sterile, pollination was done without emasculation and was normally carried out from 11.30 a.m. to 12.30 p.m.

A limited number of BC_1 plants was obtained due to high female sterility of the F_1 hybrids. To restore fertility of the sterile F_1 hybrids, we initiated chromosome doubling through colchicine treatment (0.3% colchicine + 2% dimethyl sulfoxide, 6 h) of nodal segments of the F_1 hybrids (Wong 1989). To stimulate fertilization, seed set and hybrid embryo development in producing backcross progenies, a solution of 75 ppm GA_3 (gibberelic acid) was sprayed onto pollinated panicles immediately after pollination and again a day after pollination (D.S. Brar personal communication). Abscission was prevented by spraying the pollinated panicles once daily with a solution of 75 ppm GA_3 and 75 ppm NAA (napthalene acetic acid) for 4–5 successive days (Amante-Bordeus et al. 1992).

In all of the crosses, the seeds were imperfectly developed. Most of the F_1 hybrid and BC seeds started to degenerate at approximately 2 weeks after pollination, but some others could grow till maturity on the mother plant. To obtain a sufficient number of F_1 hybrids and backcross progenies, the embryo rescue technique was applied. The seeds were removed from the glumes and surface sterilized by shaking them in 20% Clorox (commercial bleach) with a drop of Tween 20 (polyoxyethylene sorbiton monolaurate) for 15–20 min. The young embryos were then excised aseptically under a stereoscopic dissecting microscope. The excised embryos were cultured on 25% strength Murashige and Skoog's medium (Murashige and Skoog 1962) following the procedure of Jena and Khush (1984).

The cultured embryos were initially incubated in the dark at a temperature of $28^{\circ} \pm 1^{\circ}C$ until germination and then transferred to lighted incubation conditions. The young seedlings at the three-leaf stage were transferred to a liquid nutrient solution (Yoshida et al. 1976) for about 10 days before transplantation to soil.

Cytological and fertility studies

Pollen fertility of the F_1 hybrids and BC progenies was estimated by staining mature pollen grains with a staining solution developed by Alexander (1980). The stainning solution is a mixture of various constituents, namely, ethanol, malachite green, glycerol, acid fuchsin, phenol, lactic acid and distilled water. Pollen grains which stained a crimson red were considered fertile; those staining green, as sterile. At least 1000 stained pollen grains were examined under the light microscope and recorded per plant, and the percentage of fertile pollen grains was then calculated. Plants with 90% fertile pollen were classified as highly fertile; with 75–90% as fertile; with 50–74% as partly sterile; with 10–49% as sterile and less than 10% as highly sterile (Chang and Bardenas 1965).

Root-tip squashes were used to determine the chromosome number of the F_1 hybrids and backcross progenies. Root tipes were pretreated with 2 m*M* 8-hydroxyquinoline (2–3 h), fixed in Farmer's solution (3 : 1, alcohol acetic acid) at room temperature ($28^{\circ} \pm 1^{\circ}C$) for 24 h, then stored in a refrigerator until use. The fixed roots were hydrolysed in 1 *N* HCl at 60 °C for 10 min, stained for 30 min with Schiff's reagent and then squashed in a drop of acetocarmine (2%).

The young inflorescences at the appropriate stage of development were fixed in Farmer's solution fortified with ferric acetate at 4 °C for a minimum period of 24 h. The fixed spikelets were stained with Snow's alcoholic hydrochloric acid carmine stain (Snow 1963), and then the anthers were squashed in a drop of 45% acetic acid. Chromosome pairing at the first meiotic metaphase was examined under the phase-contrast microscope.

Evaluation for bacterial blight resistance

The parents and hybrid progenies were tested for their reactions to bacterial blight pathogens. The wild parent, F_1 hybrids and BC progenies were clonally propagated by growing axillary buds from nodal cuttings. These clones were used for inoculation tests.

Cultures of the Xco isolates were revived on Wakimoto's agar from stock cultures and incubated at 30°–$34\,^{\circ}C$. Inoculum was prepared by suspending the bacteria in distilled water and adjusting the optical density to 1.0 (590 nm) to give a concentration of approximately 1×10^9 cells/ml.

O. sativa parents were inoculated approximately 50 days after sowing, while clones of *O. minuta* and F_1 and BC progenies were inoculated when several tillers were available. The plants were inoculated using the clipping method described by Kauffmann et al.

(1973) The bacterial cell suspension was applied to the two youngest fully expanded leaves of each tiller by clipping 2–3 cm from the tip of the leaf using a pair of scissors dipped in the inoculum. Lesion lengths (LLs) of the leaves were measured 14 days after inoculation and scoring was done following Machmud (1978). Plants with LLs of 0–3 cm were scored as resistant (R), those with LLs of 3.1–6.0 cm were scored as moderately resistant (MR), with LLs of 6.1–9.0 cm as moderately susceptible (MS), and those with LLs of 9.1 cm or greater were scored as susceptible (S). For this evaluation study, ten leaves per plant with five replicates were inoculated with each isolate of bacteria Xco.

Isozyme analysis

Isozyme diversity at five loci (*Sdh-1*, *Pgd-1*, *Pgd-2*, *Got-1* and *Got-3*) in interspecific derived progeny (F_1, BC_1 and BC_2) was analysed using starch gel electrophoresis as described by Galzzman et al. (1988). This analysis was carried out to monitor the relative genetic contribution of *O. minuta* in the derived progeny.

Results

Production, morphology, cytology and fertility of the F_1 hybrids and BC progenies of the F_1 hybrids.

A total of 1227 (14.8%) F_1 hybrid seeds were obtained after pollinating 8282 spikelets (Table 1). The seed setting percentage ranged from 9.5% to 25.1%, depending upon the rice variety used. Seed set from the cross of 'Setanjung' × *O. minuta* was 25.1%, which was the highest, followed by the seed set from the cross of 'Mahsuri' × *O. minuta* (15.1%), 'MR84' × *O. minuta* (12.5%) and 'MR103' × *O. minuta* (9.5%).

By rescuing 10- to 14-day-old embryos and culturing them on 25%-strength MS medium, we ensured a good germination and survival of the hybrid plants. The germination percentages ranged from 91.8% to 96.4% of the embryos cultured. By culturing the seedlings in a nutrient solution prior to transplantation into soil, we were able to raise a total of 414 (84.2%) F_1 plants to maturity. The F_1s resembled *O. minuta* although in growth habit and in some morphological characters such as panicle type and length, spikelet size and stigma colour, they were intermediate between the two parents (Fig. 1).

The hybrids were uniform, vigorous, male-sterile (0.43–0.61% stainable pollen) and tillered profusely. Like the male parent, the hybrids were perennial and had long awned spikelets and well exserted panicles.

All F_1 hybrids were triploid (ABC genome) with 36 chromosomes. An examination of meiosis in these hybrids revealed the presence of a low frequency of bivalents, with an occasional trivalent and a quadrivalent (Table 2).

BC_1 progeny

In general, seed set was lower upon backcrossing (0.11% from about 40 813 spikelets pollinated) than when the parents were crossed (Table 1). Extremely low seed set (0.03%) was obtained when the triploid F_1s were backcrossed to their respective *O. sativa* parent. However, when the backcrosses were made on the colchicine-treated nodal segments, a slightly higher seed set was obtained.

Out of 44 embryos cultured, 17 BC_1 plants were obtained. Of these, 1 plant was obtained from backcrosses of the triploid F_1, 6 plants were obtained by backcrossing the fertile plant resulting from the colchicine treatment and another 10 were obtained by backcrossing the sterile colchicine-treated plants. No BC_1 plant was obtained from the cross of F_1 ('MR84' × *O. minuta*) × 'MR84' even after the pollination of 13 894 spikelets.

The morphological characteristics of the BC_1 plants appeared to be more like those of the F_1 hybrids. The plants were perennial and male-sterile (0.37–34.00% pollen stainability). The chromosome numbers of the BC_1 plants varied from 44 to 48, and the plants exhibited irregular meiosis, with mostly univalents.

BC_2 progeny

Overall, out of 4452 spikelets pollinated followed by the application of growth regulator, a total of 160 BC_2 seeds (2.47% seed set) was obtained (Table 1). Of these, 50 seeds were without embryos and had watery or clear liquid endosperm.

Table 1 Production of *Oryza sativa* × *O. minuta* F_1 hybrids and backcross progenies (OF_1 original triploid F_1, CTF_1 colchicine-treated F_1)

Cross	Spikelets pollinated (no.)	Seed set (%)	Embryos cultured (no.)	Germinated embryos (no.)	Plants obtained (no.)	Chromosome number (2n)
Mahsuri × *O. minuta*	1742	15.1	132	123	108	
Setanjung × *O. minuta*	1729	25.1	138	133	123	
MR84 × *O. minuta*	2465	12.5	110	101	89	
MR103 × *O. minuta*	2346	9.5	112	103	94	
Overall	8282	14.8	492	460	414	36
OF_1 × *O. sativa*	37080	0.03	10	6	1	
CT F_1 Mahsuri × Mahsuri	2071	0.63	13	13	10	
CT F_1 Setanjung × Setanjung	1662	1.20	20	15	6	
Overall	40813	0.11	43	34	17	44–48
BC_1 × *O. sativa*	4452	2.47	110	80	32(18)[a]	24–37

[a] Figure in parenthesis shows the number of plants with normal growth

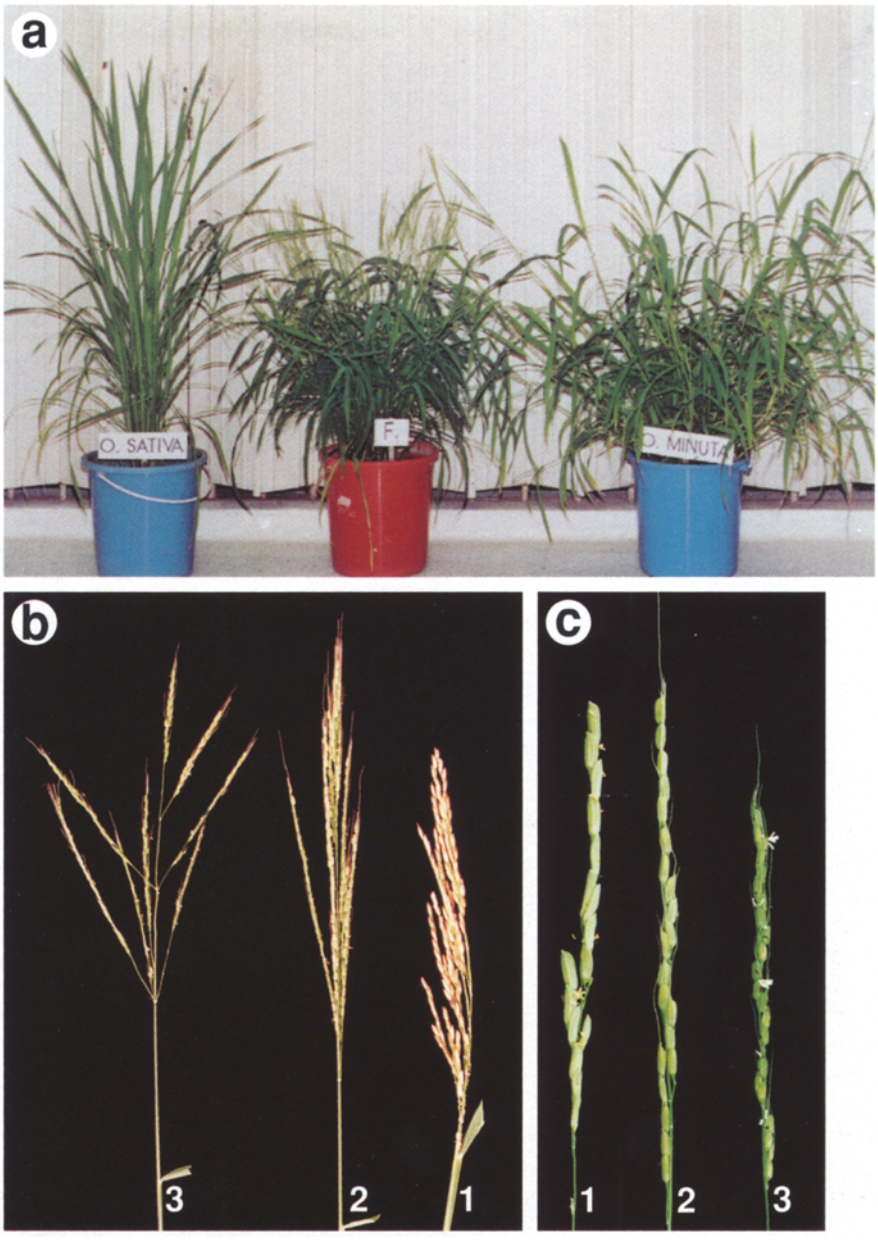

Fig. 1a–c Morphology of *O. sativa* (*1*), F_1 (*2*) and *O. minuta* (*3*). **a** Whole plant, **b** panicle, **c** spikelet

By rescuing 10- to 14-day-old embryos we obtained 32 BC_2 plants. However, of these, only 18 plants grew normally and reached the flowering stage. The rest of the plants showed abnormal growth and were stunted. All of the 18 BC_2 plants were morphologically distinct, and they differed in growth habit, height, shape and length of leaves, size of ligule, presence of absence of awns, pollen fertility (0.08–58.8%), size of spikelets and panicle length. Of these 18 plants 1 closely resembled the *O. sativa* parent (Fig. 2) and had 58.8% pollen fertility and 12.5% spikelet fertility. Some traits absent in both parents, such as purplish stem base, leaf rolling partially enclosed panicles, were observed in some of the BC_2 progenies.

Seventeen BC_2 plants had 30–37 chromosomes, and 1 had 24. Except for the 24-chromosome plant, the BC_2 plants generally showed irregular meiosis, with mostly univalents. Of the 60 pollen mother cells (PMCs) of the 24-chromosome plant observed, 60% had 12 IIs, 23.3% had 11 II + 2 I, 13.3% showed 10 II + 4 I and 3.3% had 9 II + 6 I.

Evaluation for bacterial blight resistance

Table 3 shows the ranges and means of the lengths of the lesions of the parents, *O. sativa* and *O. minuta*, and their derived progenies after inoculation with five Malaysian isolates of *Xanthomonas campestris* pv *oryzae* (Xco). The *O. minuta* parent showed a high level of resistance to the five isolates of Xco (lesion lengths of less than 1.0 cm), while amongst the *O. sativa* parents, differential reactions to every isolate was observed. 'Mahsuri' was resis-

Table 2 Mean (± SD) and range of chromosome pairing per cell at first meiotic metaphase of *Oryza sativa* X *O. minuta* hybrids

Hybrid	Plants observed (no.)	Cells observed (no.)	Chromosome pairing						Chiasmata
			I	II total	II rod	II ring	III	IV	
F_1Mahsuri	15	675	29.34 ± 3.30 (18–36)	3.31 ± 1.65 (0–9)	3.18 ± 1.65 (0–9)	0.14 ± 0.35 (0–2)	0.01 ± 0.10 (0–1)	0.001 ± 0.04 (0–1)	3.48 ± 1.73 (0–9)
F_1MR1	15	675	28.56 ± 3.26 (18–36)	3.71 ± 1.63 (0–9)	3.59 ± 1.61 (0–9)	0.12 ± 0.33 (0–2)	0.006 ± 0.08 (0–1)	0	3.84 ± 1.72 (0–9)
F_1MR84	15	675	29.75 ± 3.40 (20–36)	3.09 ± 1.68 (0–8)	3.01 ± 1.65 (0–8)	0.079 ± 0.28 (0–2)	0.021 ± 0.14 (0–1)	0.001 ± 0.04 (0–1)	3.24 ± 1.78 (0–10)
F_1MR103	15	675	29.57 ± 3.75 (16–36)	3.17 ± 1.87 (0–10)	3.09 ± 1.82 (0–9)	0.087 ± 0.31 (0–3)	0.025 ± 0.16 (0–1)	0.004 ± 0.07 (0–1)	3.32 ± 1.98 (0–13)
Overall			29.31 ± 3.37 (16–36)	3.32 ± 1.68 (0–10)	3.22 ± 1.66 (0–9)	0.11 ± 0.32 (0–3)	0.016 ± 0.12 (0–1)	0.002 ± 0.04 (0–1)	3.46 ± 1.77 (0–13)

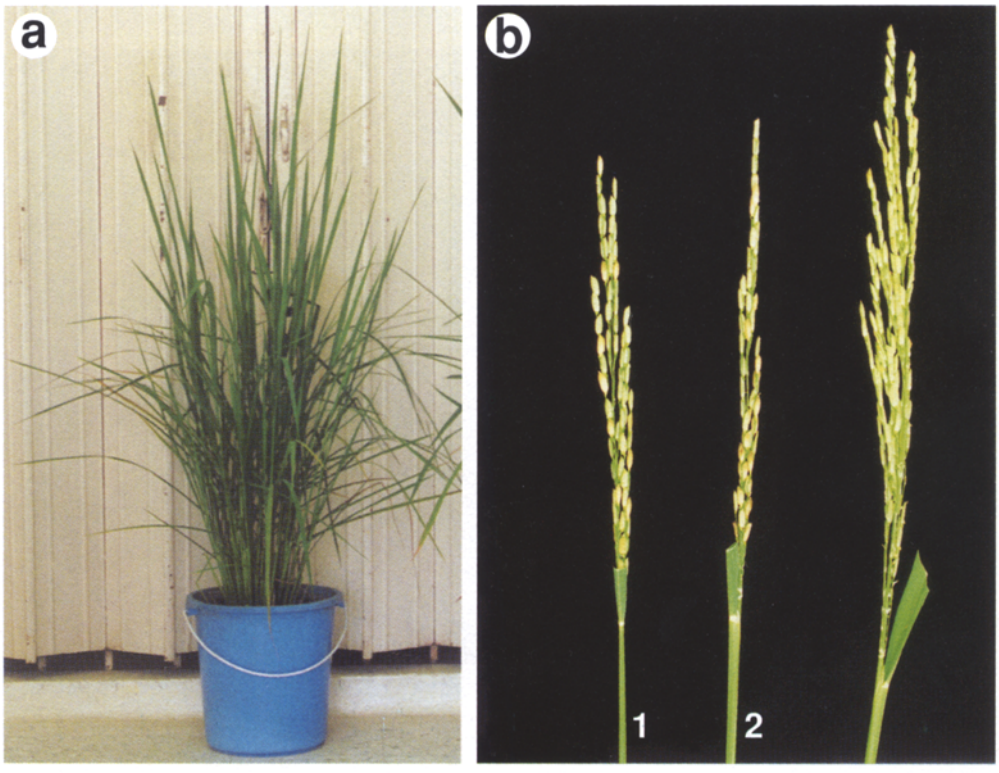

Fig. 2a, b Morphology of the 24 BC_2 plant. **a** Whole plant. **b** panicle (*1* and *2*)

tant or moderately resistant to the isolates XO66 and XO99, moderately resistant to XO319 and showed intermediate resistance to isolate XO100 and XO257. Variety 'Setanjung' was susceptible to XO100 and was resistant to the rest of the isolates. 'MR84' was moderately resistant to XO66 and XO257, moderately susceptible to XO99 and susceptible to the other two isolates. 'MR103' was resistant to XO66 and XO319, resistant to moderately resistant to XO99 and XO257 and susceptible to XO100.

All of the F_1 and BC_1 plants were resistant to the five isolates. The 'Mahsuri'-derived BC_2 progeny were all resistant or moderately resistant to the five isolates. From the 'Setanjung'-derived BC_2 progeny, 10 plants were tested, and these were either resistant or moderately resistant to isolates XO66, XO99, XO257 and XO319. For isolate XO100, 3 of the BC_2 plants were found to be moderately susceptible, and the others were moderately resistant. The 24-chromosome BC_2 plant was resistant to isolates XO66, XO99, XO257 and XO319 with mean lesion lengths of 2.60, 1.88, 2.98 and 1.98 cm, respectively, and was moderately resistant to isolate XO100 with a mean lesion length of 4.57 cm.

Table 3 Lesion lengths (cm)[a] for *O. sativa* vars 'Mahsuri', 'MR84' and 'MR103', *O. minuta* and derivatives after inculation with five isolates of *Xanthomonas campestris* pv. *oryzae*

	X066		X099		X0100		X0257		X0319	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
O. minuta	0.57	0.75	0 69	1.05	0.47	0 62	0.20	0.74	0.35	0.55
O. sativa										
Mahsuri	2.49	4.21	2.20	3.52	9.55	13.57	4.06	5.31	3.12	4.43
MR1	1.60	2.65	1.38	2.94	9.88	13.07	1 09	2.63	1.70	2 69
MR84	4.29	5.11	7.80	9 85	16 24	19.71	5.73	6.94	12.20	12.40
MR103	0.93	0 98	1.97	3.39	14.37	15.83	2.17	3.87	2.36	2 80
Derivatives										
F_1 Mahsuri	0.57	0.81	0.48	0.96	0.57	1.05	0 36	0.97	0.57	0.62
F_1 MR1	0.21	0.76	0.40	0.69	0.49	0.92	0.46	0 77	0.37	0.67
F_1 MR84	0.52	0.87	0.61	0.92	0.93	1.50	0 95	1.34	1.01	1.23
F_1 MR103	0.26	0 40	0 32	0.54	0.77	1.31	0.46	0.87	0.51	0.74
$BC_1M(n^b = 7)$	0.93	1.75	0.94	2 07	1.86	2 36	1.17	2.11	1.04	1.80
$BC_1S(n = 5)$	0.85	1.25	0.97	1.72	1.22	2.75	1.06	1 50	0.97	1.57
$BC_2M(n = 4)$	2.40	3.17	2.56	3.34	2 95	4 89	3.03	4.04	1.05	3.89
$BC_2S(n = 10)$	0.95	4.24	1.88	4.20	4.21	6.65	2.97	3.49	1.46	5.14

[a] Ranges of means from several inoculated leaves per plant
[b] *n*, the number of plants tested

Isozyme analysis

Only 'Mahsuri' and 'Setanjung' and their derived progenies were analysed for isozymes. No polymorphism was observed between 'Mahsuri' and 'Setanjung' at the five loci, but between *O. sativa* and *O. minuta*, there was polymorphism at the *Sdh-1*, *Pgd-2*, *Got-1* and *Got-3* loci (Table 4). Thus, these loci could be used as markers for monitoring the introgression of *O. minuta* genetic material in the backcross progenies. When the zymograms of the parents, F_1 and BC_1 were compared, the results showed that the F_1 and BC_1 had similar heterozygous bands for *Sdh-1*, *Pgd-2*, *Got-1* and *Got-3*. For *Sdh-1*, *Pgd-2* and *Got-3*, both parents showed a single band, but the F_1 and BC_1 had either two or three bands depending on whether the isozymes were monomers or dimers. For *Got-1*, *O. sativa* had one band, but the wild *O. minuta*, F_1 and BC_1 had three bands.

Of the 17 BC_2 plants studied, 8 were heterozygous for *Sdh-1* and *Got-3*, 11 were heterozygous for *Pgd-2* and 6 were heterozygous for *Got-1*. In the 24-chromosome BC_2 plant, none of the *O. minuta* isozyme bands was detected.

Discussion

In general, low seed set is a common problem encountered in wide hybridization. In the genus *Oryza*, interspecific hybridization, especially when involving intergenomic crosses, is normally characterized by low seed set (0–26.5%) that is commonly less than 10% (Sitch et al. 1989a). In the present study, crosses between four varieties of *O. sativa* as female parents and *O. minuta* as male parent gave seed sets between 9.5% and 25.1%, depending on the variety used. The seed sets obtained were found to be higher than those reported in the previous studies. Amante-Bordeus et al. (1992) and Sitch et al. (1989a) reported that crosses of *O. sativa* × *O. minuta* gave only 0–4.2% seed set. However, Nezu et al. (1960) obtained 21.03% seed set in just such a cross-combination. The differences observed in seed setting

Table 4 Isozyme polymorphism between *O sativa*, vars 'Mahsuri' and 'MR1' and *O. minuta* acc. no. 101141 (*F* fast mobility, *S* slow mobility, *I* intermediate activity)

Plant	Isozyme				
	Sdh-1	*Pgd-1*	*Pgd-2*	*Got-1*	*Got-3*
Mahsuri	F	F	S	F	F
Setanjung	F	F	S	F	F
O minuta	S	F	F	F, I, S	S
F_1	F, S	F	F, I, S	F, I, S	F, I, S
BC_1	F, S	F	F, I, S	F, I, S	F, I, S
BC_2(24 chromosomes)	F	F	S	F	F

between this study and the previous studies probably reflect differences in the genetic backgrounds of the *O. sativa* parents and the accession of the wild species used. According to Sitch (1990), individual accessions of some species of *Oryza* differ noticeably in crossability. For example, crosses of 'IR36' with *O. nivara* (Acc. 101973) yielded 9.1% seed set, while crosses with *O. nivara* (Acc. 103826) gave 62.2%. In crosses between three breeding lines of *O. sativa* and *O. officinalis*, Jena and Khush (1986) obtained a different percentage of seed set for each cross-combination. These results indicate that seed set is affected by the genotype of the parents used.

The interspecific hybrid seeds obtained in this study had imperfectly developed endosperm, which resulted in the abortion of the embryos at early stages of their development. Some of the seeds could develop till maturity on the mother plant regardless of the abnormality in the endosperm. However, the ability to survive was very poor, although they germinated well in the petri dish. This indicates that the degenerated endosperm affects the development of the hybrid seedlings to some extent. Embryo rescue on 25% strength MS medium was therefore helpful in ensuring the survival of the hybrids.

As expected in most intergenomic hybrids, meiotic chromosome pairing in *O. sativa* × *O. minuta* hybrids was irregular, with mostly univalents, a low frequency of bivalents and the occasional trivalent and quadrivalent. The bivalents observed were mostly rod bivalents. These results reaffirmed those obtained by previous researchers such as Nezu et al. (1960) and Li et al. (1963).

The occurrence of a low frequency of bivalents in the F_1 hybrids indicated that there is a possibility for gene transfer from the *O. minuta* genome into that of *O. sativa* through recombination. According to Nezu et al. (1960), Kihara et al. (1961) and Katayama (1966), who studied the species relationship amongst A, B, BC and CD genomes, the observed chromosome pairing in *O. sativa* × *O. minuta* hybrids are between the A genome and the B or C genomes.

In general, distant hybrids often show a high level of sterility (Khush and Brar 1988). In rice, crosses between cultivated rice and its wild relatives often show a high male sterility. The sterility can 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. According to Oka and Chang (1962), the sterility in interspecific crosses of rice can also be due to genic differences, in particular in hybrids from crosses that involve the A genome. In the present study, all hybrids from the cross of *O. sativa* × *O. minuta* showed very high pollen sterility. Observation of their spikelets at flowering showed that the anthers remained undehiscent due to the lack of fertile pollen. Similar observations were reported by Amante-Bordeous et al. (1992) for this cross-combination. A study by Chu et al. (1969) on reproductive barriers in cultivated rice species and their wild relatives reported that the fertility of the embryo sacs appeared to be similar to that of pollen grains. Our results concur with this report at the seed set obtained from backcrosses of all triploid F_1s with their respective parents was very low.

Backcrossing and alien gene transfer

Backcrossing has been an important procedure for transferring genes from distantly related species to commercially useful varieties. Repeated backcrossing to the cultivated species usually results in the restoration of fertility and stability in hybrid progenies and eliminates most or all of the wild species chromosomes. In rice, backcrossing has been successfully used to transfer several useful genes from wild species into cultivated varieties (Khush 1977; Jena and Khush 1990; Amante-Bordeous et al. 1992; Multani et. al. 1994).

In general, recovery of the phenotype of the recurrent parent requires the material to be advanced up to the BC_5 or BC_6 generation (Kalloo 1992). However, in rice wide crosses, rapid recovery of the phenotype of the recurrent parent has been observed. For example, Amante-Bordeous et al. (1992) and Multani et al. (1994) have obtained diploid plants that resemble the recurrent *O. sativa* parents after two backcrosses. They suggested that the rapid recovery of the recurrent parent phenotype was a result of restricted recombination between the parental genomes as shown by the low frequency of rod bivalents in the meiosis of the F_1 plant.

In this study 1 diploid plant ($2n = 24$) was obtained after the second backcross. The plant resembled *O. sativa*, and was partially fertile with 58.8% pollen stainability and 12.5% seed set under self-pollination. On the basis of the morphological similarity to *O. sativa*, fertility and the behaviour of the chromosome pairing, which showed 12 bivalents in 60% of the PMCs observed, some genetic material was transferred from *O. minuta*, probably through recombination. On the other hand, the isozyme study showed that none of the *O. minuta* isozyme bands (*Sdh-1*, *Pgd-2*, *Got-1*, and *Got-3*) was detected in the 24-chromosome plant, indicating that the transfer did not involve any of the isozyme loci used as markers.

However, resistance to bacterial blight was also transferred from *O. minuta* to *O. sativa*. Because the *O. sativa*, var 'Setanjung', was susceptible to isolate XO100, the moderately resistant reaction of the 24-chromosome indicates that resistance to this isolate was transferred from *O. minuta*. Since the parent 'Setanjung' is known to possess the resistance gene *Xa-4*, further work with the 24-chromosome plant will focus on characterizing the resistance gene transferred and also in reducing the extraneous alien DNA transferred. The resistance spectrum of this plant will be tested with all available isolates.

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References

Alexander MP (1980) A versatile stain for pollen, fungi, yeast and bacteria. Stain Technol 55:13–18

Amante-Bordeous AD, Sitch LA, Nelson RJ, Dalmacio RD, Oliva NP, Aswidinnoor H, Leung H (1992) Transfer of bacterial blight and blast resistance from the tetraploid wild rice *Oryza minuta* to cultivated rice, *Oryza sativa*. Theor Appl Genet 84:345–354

Chang TT, Bardenas FA (1965) The morphology and varietal characteristics of the rice plant. Technical Bulletin 4. International Rice Research Institute (IRRI), Los Banos, Philippines

Chen YH, Othman O, Ong HS, A'aini AR, Lim KH, Azlan S, Abdul Wahab H (1990) Varieti Padi Baru MR103 Teknol Padi 6:25–29

Chu YE, Morishima H, Oka HI (1969) Reproductive barrier distributed in cultivated rice species and their wild relatives. Jpn J Genet 44:207–223

Glaszmann JC, Delos Reyes BG, Khush GS (1988) Electrophoretic variation of isozymes in plumules of rice (*Oryza sativa* L.) – a key to the identification of 76 alleles at 24 loci. IRRI Res Paper Ser No. 134

Heinrichs EA, Medrano FG, Rapusas HR (1985) Genetic evaluation for insect resistance in rice. International Rice Research Institute, Manila, Philippines

International Rice Research Institute (1993) Annual Report for 1992. Manila, Philippines

Jena KK, Khush GS (1984) Embryo rescue of interspecific hybrids and its scope in rice improvement. Rice Genet Newsl 1:133–134

Jena KK, Khush GS (1986) Production of monosomic alien addition lines of *Oryza sativa* having a single chromosome of *O. officinalis*. In: Rice Genetics I. International Rice Research Institute, Manila, Philippines, pp 119–208

Jena KK, Khush GS (1990) Introgression of genes from *Oryza officinalis* Well ex Watt to cultivated rice, *O. sativa* L. Theor Appl Genet 80:737–745

Kalloo G (1992) Utilization of wild species. In: Kalloo G, Chowdhury JB (eds) Distant hybridization. Springer, Berlin Heidelberg, New York, pp 149–166

Katayama T (1966) Cytogenetical studies on the genus *Oryza*. 2. Chromosome pairing in the interspecific hybrid with the ABC genome. Jpn J Genet 41:309–316

Kaufmann HE, Reddy APK, Hsieh SPY, Nenca SD (1973) An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis Rep 57:537–541

Khush GS (1977) Disease and insect resistance in rice. Adv Agron 29:265–341

Khush GS, Brar DS (1988) Wide hybridization in plant breeding. In: Zakri AH (ed) Plant breeding and genetic engineering. SABRAO, Malaysia, pp 141–188

Khush GS, Bacalangco E, Ogawa T (1990) A new gene for resistance to bacterial blight from *O. longistaminata*. Rice Genet Newsl 7:121–123

Kihara H, Nezu M, Katayama C (1961) Genome analysis in the genus *Oryza*. II. Annu Rep Natl Inst Genet Jpn 11:40–41

Li HW, Chen CC, Wang TS, Wuu KD (1963) Cytogenetical studies of *Oryza sativa* L. and its related species. 4. Interspecies crosses involving *Oryza australiensis* with *O. sativa* and *O. minuta*. Bot Bull Acad Sinica 4:65–74

Machmud M (1978) Variation in virulence of *Xanthomonas oryzae* and effect of mixed inoculum on lesion development. MSc thesis, University of the Philippines at Los Banos, Philippines

Multani DS, Jena KK, Brar DS, De los Reyos BG, Angeles ER, Khush GS (1994) Development of monosomic alien addition lines and introgression of genes from *O. australiensis* Domin. to cultivated rice *O. sativa* L. Theor Appl Genet 88:102–109

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–496

Nezu M, Katayama TC, Kihara H (1960) Genetic study of the genus *Oryza*. I. Crossability and chromosomal affinity among 17 species. Seiken Jiho 11:1–11

Oka HI, Chang WT (1962) Rice varieties intermediate between wild and cultivated forms and the origin of the japonica type. Bot Bull Acad Sin 3:109–131

Othman O, Alias I, Hadhim K (1986) Rice varietal development in Peninsular Malaysia. MARDI Report No. 109. Institut Penyelidikan dan Kemajuan Pertanian Malaysia (MARDI)

Othman O, Guok HP, Khalid H (1994) Genetics and rice breeding. In: Koh CL (ed) Proc 1st Natl Congr Genet. Genetic Society of Malaysia, Kuala Lumpur Malaysia, pp 93–96

Sitch LA (1990) Incompatibility barriers operating in crosses of *O. sativa* with related species and genera In: Gustafson JP (ed) Gene manipulation in plant improvement II. (19th Stadler Genetics Symposium). Plenum Press, New York, pp 77–94

Sitch LA, Romero GO (1990) Attempts to overcome prefertilization incompatibility in interspecific and intergeneric crosses involving *Oryza sativa* L. Genome 33:321–327

Sitch LA, Dalmacio RD, Romero GO (1989a) Crossability of the wild *Oryza* species and their potential use for improvement of cultivated rice (*Oryza sativa* L.) Rice Genet Newsl 6:58–60

Sitch LA, Amante-Bordeous AD, Dalmacio RD, Leung H (1989b) *Oryza minuta*, a source of blast and bacterial blight resistance for rice improvement. In: Mujeeb-Kaji A, Sitch LA (eds.) Reviews of advances in plant biotechnology, 1985–1988: 2nd Intl Symp Genet Manipulation Crops. CIMMYT, Mexico DF, Mexico, pp 31–321

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosome in squash preparations. Stain Technol 38:9–13

Wong CK (1989) A new approach to chromosome doubling for haploid rice plants. Theor Appl Genet 77:149–151

Yoshida S, Forno OA, Cock JH, Gomez KA (1976) Laboratory manual for physiological studies of rice, 3rd edn. International Rice Research Institute, Los Banos, Philippines