Abnormal Embryo Development and Efficient Embryo Rescue in Interspecific Hybrids, *Oryza sativa* × *O. minuta* and *O. sativa* × *O. officinalis*

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Interspecific hybrids between cultivated rice (*Oryza sativa*, 2n = 24, AA) and two wild species (*O. minuta*, 2n = 48, BBCC and *O. officinalis*, 2n = 24, CC) have been produced by cross pollination. The embryo development in these interspecific hybrids was observed to study the deterioration features in detail. All hybrids showed either abnormal embryo development with degenerating endosperm or embryo retardation itself. Abnormally developed embryos were observed by the end of 7 days after pollination and the percentage of abnormally developed embryos increased from 7 to 14 DAP. The embryos were excised at 7 to 14 DAP and cultured on MS medium supplemented with 1, 3 and 5 mg/l benzyl-aminopurine, 1 g/l casein hydrolysate, 0.8% agar and 3% sucrose. The optimal time for rescue turned out to be 11-14 DAP. There was a significant difference (P = 0.05) in the regeneration efficiency rate with rescue time but no significant difference with of culture medium in these cross combinations. The F₁ hybrids were morphologically intermediate between their parents. All F₁ hybrid plants thus obtained were completely sterile. Chromosome doubling was attempted by treating F₁ hybrid tillers with colchicine to overcome their sterility. The morphology of the colchicine-treated F₁ plants closely resembled the female parent and fertility.

Key Words: *Oryza sativa*, *O. minuta*, *O. officinalis*, interspecific cross, embryo rescue.

Introduction

The wild *Oryza* species are a rich source of useful traits for rice improvement, such as resistance to insect-pests and diseases (Heinrichs *et al.* 1985, Sitch 1990). A number of important traits have been transferred from wild species into cultivated species by interspecific hybridization (Amante-

Bordeos *et al.* 1991, 1992, Jena and Khush 1989, 1990, Khush *et al.* 1990, Multani *et al.* 1994, Pongtongkam *et al.* 1997, Suputtitada *et al.* 1994, 2000). Hybridization between cultivated rice (AA genome) and wild species sharing the same AA genome, such as *O. nivara*, *O. rufipogon* and *O. longistaminata* has been especially successful. However, more remote hybridization between *O. sativa* and the wild species, such as *O. minuta* (BBCC), *O. officinalis* (CC) or *O. brachyantha* (FF) are difficult due to failure in embryo development. This inability of remote hybrids to undergo normal sexual reproduction might be overcome by embryo rescue, because the hybrid plants from remote parents have been achieved by embryo rescue (Amante-Bordeos *et al.* 1991, 1992, Jena and Khush 1989, Multani *et al.* 1994, Pongtongkam *et al.* 1997, Suputtitada *et al.* 1994, 2000).

The goal of our investigation was to identify features occurring at the post-fertilization stage of the interspecific cross of *O. sativa* cv. RD 6 (2n = 24, AA), a Thai rice as female parent and two species of wild rice, *O. minuta* (2n = 48, BBCC) and *O. officinalis* (2n = 24, CC), bearing resistance to brown plant hopper, green leaf hopper, white-black plant hopper blast and bacterial blight (Sitch *et al.* 1989, Vanghan and Sitch 1991) as the male parent and to rescue hybrid embryos with high efficiency. An attempt was also made to obtain fertile plants through colchicine treatment.

Materials and Methods

The maternal spikelets were emasculated by dipping in water of 42°C for 10 minutes before anthesis (6-8 a.m.). Pollination was performed with fresh pollen of the wild species at their anthesis. Cross-pollinated spikelets were covered with a glassine bag and collected at 1, 3, 5, 7, 9, 11 and 14 days after pollination (DAP). Normarski's differential interference contrast (DIC) microscopy was used for zygotic and post zygotic analysis as described by Suputtitada *et al.* (2000). Fertilization rate, embryo development and endosperm development were also recorded.

Hybrid embryos were detached from spikelets at 7-14 DAP. They were dissected with forceps, and were surface-sterilized with 70% ethanol for 2 minutes followed by 0.5%

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sodium hypochloride for 10-15 minutes and by rinsing with sterile water for 3 times 1 minute each. We cultured the excised embryos on MS agar medium (Murashige and Skoog, 1962) supplemented with benzylaminopurine at 1, 3 and 5 mg/l, caseine hydrolysate 1 g/l and 3% sucrose at $25 \pm 2^\circ\text{C}$. The seedlings were tillered on suitable medium with 0.3% activated charcoal for about 4 weeks (Suputtitada *et al.* 1994). Some parts of the plants were transplanted to grow in natural conditions, other parts were treated with colchicine to induce chromosome doubling. Four concentrations (0.00, 0.01, 0.03, 0.05%) of colchicine were added to the induction medium (MS liquid medium supplemented with benzylaminopurine at 1 mg/l, caseine hydrolysate 1 g/l and 3% sucrose). The F_1 plants were incubated for 3, 5 and 7 days on this medium at $25 \pm 2^\circ\text{C}$ and then transferred to a colchicine-free medium for further culturing. The cultures were then incubated for 1 month, and thereafter transplanted to soil. The fertility, plant characteristics and chromosome number were studied.

Chromosome number was studied using root tips pre-treated with distilled water for 3 h at 4°C , followed by immersion in a 0.03% 8-hydroxy-quinolinol solution for 3 h at 25°C and fixed with acetic alcohol (1 : 3) for 24 h at 25°C . After fixation, the root tips were treated with an enzyme solution containing 1.0% Pectolyase Y23 and 2% Cellulase Onozuka RS (pH 4.0) for 3 h at 25°C and subsequently washed 3 times with distilled water. After treatment with 1 N HCl for 10 min, the root tips were squashed in 1% acetocarmine solution and analyzed microscopically.

Results and Discussion

Embryo development

Embryo development of self-pollinated *O. sativa* cv. RD 6 was used as a control for embryo development (Table 1). All embryo sacs contained a zygote at 1 DAP. The average diameter of the zygotes was 20 μm . In two thirds of these embryo sacs two endosperm nuclei were visible, the remaining one third had three to twelve nuclei. Until 2 DAP, the fertilized egg cell still remained at the zygote stage. The zygote started to divide after the endosperm nuclei had divided several times. At 3 DAP, the zygote developed into the proembryo stage (Fig. 1A). The average diameter of the proembryos was 30 μm . The endosperm nuclei were located at the periphery of the embryo sac within 7 DAP. At 7 DAP, the proembryo developed into the globular stage (Fig. 1B). However, most globular embryos were found from 9 DAP on and the average diameter of globular embryos was 50 μm . On the 11 DAP, the embryos of *O. sativa* had grown into fully developed structures (Fig. 1C).

In cross-pollinations between *O. sativa* $\times$ *O. minuta* and *O. sativa* $\times$ *O. officinalis*, more than 250 embryo sacs of each were observed (Table 1). Fully developed embryos, similar to those in Fig. 1C, were not observed at 14 DAP in either cross-combination, even though retarded embryos were still alive. Embryo sacs contained either degenerated or retarded embryo. Both hybrid embryo sacs of *O. sativa* $\times$ *O. minuta* and *O. sativa* $\times$ *O. officinalis* contained a zygote and primary endosperm at 3 DAP (Fig. 1D). The remainings of both combinations were still at the egg or zygotic stage. At 7 DAP, the hybrid embryo sacs only divided to multiple

Table 1. Embryo development in self- and cross-pollinated *Oryza* species

Cross combination and developmental stage of embryo	Days after pollination (DAP)						
	1	3	5	7	9	11	14
<i>Self-pollination of O. sativa</i>							
Zygote	20 (100) ¹⁾	24 (61.5)	2 (4.8)	0	0	0	0
Proembryo	0	15 (38.5)	40 (95.2)	19 (48.7)	3 (7.9)	0	0
Globular	0	0	0	20 (51.3)	35 (92.1)	23 (59.0)	0
Fully differentiated	0	0	0	0	0	16 (41.0)	55 (100)
Total	20	39	42	39	38	39	55
<i>O. sativa</i> $\times$ <i>O. minuta</i>							
Zygote	18 (100)	25 (67.6)	30 (83.3)	24 (58.5)	13 (34.2)	1 (2.5)	0
Proembryo	0	0	0	0	7 (18.4)	10 (25.0)	2 (3.8)
Globular	0	0	0	0	0	8 (20.0)	18 (34.0)
Fully differentiated	0	0	0	0	0	0	0
Aborted	0	12 (32.4)	6 (16.7)	17 (41.5)	18 (47.4)	21 (52.5)	33 (62.2)
Total	18	37	36	41	38	40	53
<i>O. sativa</i> $\times$ <i>O. officinalis</i>							
Zygote	18 (100)	24 (65.9)	29 (81.1)	22 (54.3)	10 (26.3)	0	0
Proembryo	0	0	0	0	8 (21.0)	11 (28.1)	0
Globular	0	0	0	0	0	6 (15.6)	18 (34.5)
Fully differentiated	0	0	0	0	0	0	0
Aborted	0	13 (34.1)	7 (18.9)	19 (45.7)	20 (52.7)	23 (56.3)	35 (65.5)
Total	18	37	36	41	38	40	53

¹⁾ Number of embryo (percentage)

Fig. 1. Comparative development of embryo sac and embryo in self- and cross-pollinations at 3, 7 and 11 DAP. Normal development of proembryo at 3 DAP. (A). globular embryo at 7 DAP (B). fully differentiated embryo at 11 DAP (C). shoot apex (s), root apex (r) and epiblast (e). Abnormal development of *O. sativa* × *O. minuta* shows fertilized egg (small arrow) and primary endosperm nucleus (big arrow) at 3 DAP (D). multiple endosperm nuclei (arrows) at 7 DAP (E). still viable embryo at 11 DAP (F). Bar = 10 µm in Fig. 1A, 1B and 1F, Bar = 20 µm in Fig. 1D, Bar = 30 µm in Fig. 1E, bar = 100 µm in Fig. 1C.

endosperm nucleus and embryo development was delayed (Fig. 1E). At that time, the proembryos of control plants had developed to the globular stage. Globular stage embryos were found in both, *O. sativa* × *O. minuta* (Fig. 1F) and *O. sativa* × *O. officinalis*, at 11 DAP.

When the developments in both interspecific combinations were compared with the self-pollinated *O. sativa*, retardation of embryo development and/or later embryo de-

generation was obvious (Table 1). The interspecific hybrid embryo in both combinations showed a slower growth than that of the selfed one and its growth mostly stopped at the zygote and proembryo stage. At 11 DAP, the selfed embryo was usually a fully differentiated embryo while the interspecific hybrid embryo was just at the globular stage. Focussing on early developmental stages, we tried to elucidate the degradation procedure. As shown in Table 1, embryo sacs of

O. sativa × *O. minuta* and *O. sativa* × *O. officinalis* remained as single cells, unfertilized or zygotic at 3 DAP, for 67.6% and 65.9%, respectively. Development of embryos at 5 DAP was also delayed and sometimes even stopped short of 7 DAP. The percentage of aborted embryos increased from 7 to 14 DAP by more than 60%. The endosperm failed to divide and degenerated by the end of 7 DAP. In both cross-combinations, we found that embryo development was much slower than that of the control and its growth mostly stopped at the globular stage at 11-14 DAP, while the control embryos had developed to full maturity. Several hybrid embryos were slimmed in shape and collapsed. This type of failure of embryo development occurred during endosperm formation. In some cases, an embryo could develop for several days in the absence of endosperm, but the differentiation of the organ had stopped.

Endosperm failed to divide and started to degenerate and later had only a few cells around the embryo. Some embryo sacs continued to develop with a small endosperm and some embryo sacs contained embryos without endosperm. In general, the endosperm is a tissue that provides nutrients to the developing embryo. After the degeneration of endosperm, the embryo is no longer supplied with nutrients. This deficiency causes embryo starvation and inhibition of further differentiation, causing embryo abortion. On the other hand, Cooper and Brink (1944) suggested that abnormal mitotic behavior of the endosperm nuclei causes seed collapse in Gramineae.

However, nutritional deficiency and abnormal mitotic behaviour of the endosperm nuclei could be due to problems

of genetic incompatibility between two remote species. Chu and Oka (1970) reported that the F₁ embryos and endosperm begin to deteriorate at about 6 DAP in interspecific hybridization between *O. sativa* and *O. longistaminata*. When *O. longistaminata* was used as maternal parent, hybrid embryos failed to develop after 3 DAP. The authors said that these observations could be explained by the presence of a barrier controlled by a set of complementary dominant lethal genes. In genetic experiments, Sano and Kobayashi (1996) revealed that the incompatibility reaction is accomplished by factors from both the female and the male parent. Our results showed that the embryo abortion occurred at the early stage of embryo development hence they could not continue to differentiate. It seemed to be they could not develop in the absence of nutrients from endosperm and the consequence of genetic incompatibility caused both embryo and endosperm failed to develop.

Embryo culture

In total, 796 and 768 florets of *O. sativa* were pollinated with pollen grains of *O. minuta* and *O. officinalis*, respectively. Sixty seven florets with embryos were obtained in *O. sativa* × *O. minuta* and 66 florets in *O. sativa* × *O. officinalis* (Table 2). Hybrid embryos of 7 to 14 DAP were cultured on the medium. The percentage of embryos detached from maternal plants decreased with the increase of DAP in both cross-combinations as shown in Table 2. The causes due to endosperm were failure to divide and degeneration. In this experiment, we cultured embryos on MS medium supplemented with 1, 3 and 5 mg/l of BAP, notated as C₀, C₁ and

Table 2. Efficiency of embryo rescue in interspecific hybrids, *O. sativa* × *O. minuta* and *O. sativa* × *O. officinalis*

Embryo excised (DAP)	No. of pollination florets (A)	No. of detached embryos (B)	B/A (%)	Medium	No. of inoculated embryos (C)	No. of regenerated plants (D)	Regeneration efficiency (D/C) (%)
<i>O. sativa</i> × <i>O. minuta</i>							
7-10	414	40	9.7	C ₀ ¹⁾	12	3	25.0
				C ₁ ²⁾	13	4	30.0
				C ₂ ³⁾	15	6	40.0
				Total	40	13	32.5
11-14	382	27	7.1	C ₀ ¹⁾	7	5	71.4
				C ₁ ²⁾	9	5	55.6
				C ₂ ³⁾	11	5	45.5
Total	796	67		Total	27	15	55.6
<i>O. sativa</i> × <i>O. officinalis</i>							
7-10	404	39	9.7	C ₀ ¹⁾	12	2	16.7
				C ₁ ²⁾	13	4	30.8
				C ₂ ³⁾	14	4	28.6
				Total	39	10	25.6
11-14	364	27	7.4	C ₀ ¹⁾	9	5	55.6
				C ₁ ²⁾	9	4	44.4
				C ₂ ³⁾	8	4	50.0
Total	768	66		Total	26	13	50.0

¹⁾ MS medium containing 1 mg/l benzylaminopurine and casein hydrolysate 1 g/l

²⁾ MS medium containing 3 mg/l benzylaminopurine and casein hydrolysate 1 g/l

³⁾ MS medium containing 5 mg/l benzylaminopurine and casein hydrolysate 1 g/l

C₂ medium, respectively. After one week, some immature embryos germinated. Regeneration efficiency ranged from 16.7% to 71.4% for both interspecific cross combinations. The average ratios of regeneration of 7-10 DAP culture were 32.5% and 25.6% for *O. sativa* × *O. minuta* and *O. sativa* × *O. officinalis*, respectively. These ratios increased to 50.0% and 55.6% at 11-14 DAP. In three types of medium, although the highest regeneration efficiency was observed in C₀ medium, there was no significant difference among the three types of culture medium ($F = 0.24$, $P = 0.05$ in *O. sativa* × *O. minuta* and $F = 0.06$, $P = 0.05$ in *O. sativa* × *O. officinalis*). From comparison of the regeneration efficiency between 7-10 DAP and 11-14 DAP, there was no significant difference between the time to rescue in both combinations ($F = 9.11$, $P = 0.05$ in *O. sativa* × *O. minuta* and $F = 13.12$, $P = 0.05$ in *O. sativa* × *O. officinalis*). It is concluded that the time point of rescue had a significant effect for the survival of the embryo. The optimal time to excise embryos for rescue was 11-14 DAP in both cross-combinations. These findings suggest that it might be able to rescue the embryo at the globular stage.

After culture for 3 weeks, phenolic compounds were found released in the medium. Therefore, the plants were transferred to fresh medium containing 0.3% activated charcoal to absorb phenolic compounds (Suputtitada *et al.* 1994). The regenerated plants developed well within 4 weeks and were transplanted to soil. Transplanted plants were easily acclimatized to the natural conditions. The F₁ hybrids were morphologically intermediate between their parents, such as panicle type and length, floret size and stigma color. However, they resembled the male parent in decending angle of flag leaf, presence of awn and shattering habit. The F₁ hybrids had vigorous and profuse tillers (Fig. 2) but were completely sterile. The sterility results from genetic differences in structure and number of chromosomes.

Chromosome doubling

Chromosome doubling was attempted by treating F₁ hybrid tillers with colchicine to overcome their sterility. The young F₁ hybrid plants treated with 0.01%, 0.03% and 0.05% colchicine for 3, 5 and 7 days showed different responses on the culture medium. The percentage of viability of the colchicine treated F₁ plants was decreased according to the increase of colchicine concentration and duration of treatment and ranged from 10.0% to 95.0% for both cross combinations as shown in Table 3. In both cross-combinations, the plants treated with 0.01% colchicine for 3 days survived better than those given other treatments. The highest percentage of viability of the colchicine-treated F₁ plants was 90.0% and 95.0% for *O. sativa* × *O. minuta* and *O. sativa* × *O. officinalis*, respectively. However, those treated with 0.05% colchicine for 7 days failed to survive in both cross-combinations. After transplanting into soil some tillers of the colchicine-treated F₁ plants produced panicles and seeds while some produced only panicles but set no seed. The morphology of the colchicine-treated F₁ plants were closely

Fig. 2. Morphology of rice plant type at tillering stage. A. *O. sativa*, B. *O. minuta*, C. F₁ hybrid of *O. sativa* × *O. minuta*

resembled those of the female parent, such as plant type, panicle type and stigma color. The F₁ hybrid plants not treated with colchicine were used as a control. They had a morphology intermediate between their parents and were completely sterile. This result implied that colchicine could change the morphology of the plant. The percentage of seed set ranged from 1.4% to 6.5% for both cross-combinations (Table 4). The highest percentage of seed set was observed in the plants treated with 0.01% colchicine for 5 days of

Table 3. Percentage of viability of the colchicine-treated F₁ plants of interspecific hybrids, *O. sativa* × *O. minuta* and *O. sativa* × *O. officinalis*

Colchicine treatment		No. of plants treated	No. of viable plants	Viability (%)
Duration (days)	concentration (%)			
<i>O. sativa</i> × <i>O. minuta</i>				
3	0.01	20	18	90.0
	0.03	20	11	55.0
	0.05	20	8	40.0
5	0.01	20	12	60.0
	0.03	20	7	35.0
	0.05	20	3	15.0
7	0.01	20	3	15.0
	0.03	20	2	10.0
	0.5	20	—	—
<i>O. sativa</i> × <i>O. officinalis</i>				
3	0.01	20	19	95.0
	0.03	20	13	65.0
	0.05	20	9	45.0
5	0.01	20	10	50.0
	0.03	20	5	25.0
	0.05	20	5	25.0
7	0.01	20	4	20.0
	0.03	20	3	15.0
	0.5	20	—	—

Table 4. Percentage of seed set in the colchicine-treated F₁ plants of interspecific hybrids, *O. sativa* × *O. minuta* and *O. sativa* × *O. officinalis*

Colchicine treatment		No. of transplanted plants	No. of fertile plants	Percentage of fertile plants	Percentage seed set in fertile plants
Duration (days)	concentration (w/v, %)				
<i>O. sativa</i> × <i>O. minuta</i>					
3	0.01	20	4	20.0	2.3
	0.03	11	1	9.1	5.2
	0.05	7	1	14.3	3.4
5	0.01	19	3	15.8	6.3
	0.03	10	1	10.0	3.4
	0.05	10	1	10.0	2.4
7	0.01	9	1	11.1	3.2
	0.03	4	1	25.0	1.4
<i>O. sativa</i> × <i>O. officinalis</i>					
3	0.01	25	5	20.0	3.5
	0.03	18	3	16.7	6.0
	0.05	13	3	23.1	4.2
5	0.01	15	4	26.7	4.2
	0.03	9	2	22.2	6.5
	0.05	7	1	14.3	3.6
7	0.01	7	1	14.3	3.4
	0.03	4	1	25.0	3.9

O. sativa × *O. minuta* and 0.03% colchicine for 5 days of *O. sativa* × *O. officinalis*. However, the seeds were slightly smaller than the *O. sativa* seeds.

In this experiment, cytological analysis indicated that the F₁ hybrids of *O. sativa* × *O. minuta* were allotriploid of the ABC genome (2n = 36) and F₁ hybrids of *O. sativa* × *O. officinalis* were allodiploid of the AC genome (2n = 24) so they were all sterile. After doubling the chromosome num-

ber by colchicine treatment, they became allohexaploid of AABBCC genome (2n = 72) and allotetraploid of the AACC genome (2n = 48), respectively, and produced gametes having a complete set of chromosome. These plants were partially fertile and produced seed.

Our results indicated that colchicine treatment of the hybrids could make them fertile. The seeds of the colchicine-treated F₁ plants had normal growth, profuse tillers, panicles

and seed. Therefore, our next challenge is to cross them back to cultivated rice to make introgressive hybridization.

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