GENETIC AND HISTOLOGICAL EVIDENCE FOR MICROSPORE ORIGIN OF ANTHER-DERIVED PLANTS OF RICE[1]

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Abstract: Anthers of rice (*Oryza sativa* L., $2n=24$) heterozygous for the waxy gene (wx^{+}/wx) were cultured on medium supplemented with 2,4-D and with NAA and kinetin. Histological observation of the anthers thus cultured revealed that only the microspores underwent divisions leading to callus formation; the somatic tissues degenerated or remained quiescent. Upon transfer of callus to the differentiation media, plants with ploidy levels ranging from haploid to tetraploid were regenerated. Staining pollen grains with I_2-KI showed that all the diploid and tetraploid plants were homozygous. Furthermore, in both ploidy levels the ratio of waxy to nonwaxy fits the expected 1:1 segregation ratio. Thus histological and genetic evidence indicates that all anther-derived plants obtained in this study are of microspore origin.

INTRODUCTION

Anther cultures of rice (*Oryza sativa* L., $2n=24$) have given rise to plants of various ploidy levels ranging from haploid to octoploid (Chen and Lin, 1981). Genetic studies on origin of the nonhaploids have not been in full agreement. Using two simple recessive traits as markers for genetic analysis, Chen *et al.* (1982) showed that all diploids produced from anthers of hybrid rice were homozygous. However, other investigators found that certain fractions of anther-derived diploids were heterozygous as determined by isozyme patterns (Wu and Kiang, 1979) or were segregating for quantitative characters in self-fertilized progeny (Woo *et al.*, 1973; Chen and Li, 1978). These results were interpreted by Woo *et al.* (1973) and Wu and Kiang (1979) to indicate that in addition to microspores somatic tissues of the anther had also been induced to proliferate. The callus-induction medium used by Chen *et al.* (1982) was supplemented with α-naphthaleneacetic acid (NAA) and kinetin, whereas those used by other investigators contained 2,4-dichlorophenoxyacetic acid (2,4-D), an auxin which has been shown to be very effective for callus induction from rice somatic tissues (Wu and Li, 1871; Yamada, 1977). Production of heterozygous diploids from rice anther culture, therefore, may be the result of presence of 2,4-D in the culture medium. To test this possibility, we cultured anthers of rice heterozygous for the waxy gene on medium supplemented with 2,4-D and with other plant hormones, studied histology of the anthers during culture, and determined genotypes of anther-derived plants. Results of this study are presented herein.

MATERIALS AND METHODS

Plant material

Plants of *Oryzae sativa* L. cv. Hsinchu 4 homozygous for the recessive allele of the waxy

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gene were used as seed parents in matings with plants of A6, an anther-derived pure line bearing the dominant allele of this gene. Anthers of the F_1 (wx^+/wx) at the mid-uninucleate microspore stage were used as explants for callus induction and plant regeneration.

Anther culture

Two culture methods were employed. The basal media and culture conditions in the two methods were identical, and only the hormonal component was different (Table 1). A detailed procedure for rice anther culture has been described previously (Chen, 1977, 1978).

Table 1. Media used for rice anther culture

	Method 1	Method 2
Callus induction:		
Basal medium:	Modified MS* (6% sucrose)	Modified MS* (6% sucrose)
Additives:	NAA 4 mg/l kinetin 2 mg/l	2,4-D 2 mg/l
Plant regeneration:		
Basal medium:	MS (3% sucrose)	MS (3% sucrose)
Additives:	NAA 1 mg/l kinetin 4 mg/l	None

* The major inorganic salts of Murashige and Skoog (1962) medium were reduced to half of original strengths.

Maintenance of anther-derived plants

Test-tube plantlets were first transplanted to plastic cups containing sterilized soil and kept in a condition of high humidity and relatively low temperature (20 to 25°C). When the young plants were approximately 15 cm high, they were transplanted to the field. In cases where two or more plantlets emerged from callus of an anther, only one was randomly transplanted and raised to maturity.

Determination of chromosome number

Root tips were collected during transplanting or later in the field. They were treated in 0.002 M 8-hydroxyquinoline at 18 to 20°C for 3 h, fixed in ethanol-glacial acetic acid (3:1) overnight, stained in Feulgen reagent for 1 to 2 h, and then treated with 5% pectinase (ICN Pharmaceuticals, Inc.) for 2 h. In preparation of slides for chromosome counts, root tips were squashed in diluted propiono-carmine.

Determination of genotype

Genotypes of anther-derived diploid and tetraploid plants and of plants in the F_2 progeny were determined by observation of staining reaction of the mature pollen grains in I_2–KI solution. Genotypes of anther-derived haploid and triploid plants could not be determined because of sterility.

Histological procedures

Anthers were collected at intervals of 0, 7, 14, and 21 days after culture. They were fixed in formalin-acetic acid-alcohol, dehydrated in a tertiary-butyl alcohol series, and embedded in paraffin. Serial sections were cut at 10 μm, stained in Feulgen reagent and counterstained in fast green in 95% ethanol.

RESULTS AND DISCUSSION

The frequencies of callusing anthers obtained by the two culture methods were not markedly different (Table 2). However, callus initiated and cultured on media supplemented with NAA and kinetin (method 1) appeared to be more capable of plant regeneration and to produce less albino plants than callus initiated on medium containing 2, 4-D and subsequently transferred to medium without plant hormones (method 2) (Table 3). The low morphogenetic potential of callus in method 2 may be attributed to presence of 2, 4-D in the callus-induction medium and/or absence of kinetin in both the callus-induction and plant-regeneration media.

Table 2. Callus formation from cultured anthers of rice

Culture method	No. of anthers cultured	No. of anthers producing callus	Percent anthers producing callus
1	3900	1513	38.8
2	4000	1265	31.6

Table 3. Plant regeneration from anther callus of rice

Culture method	No. of callus cultured	No. of callus producing plants					Green/albino plants
		Green	Albino	Green+albino	Total	%	
1	1370	332	582	88	1002	73.1	0.63
2	976	97	333	32	462	47.3	0.35

Ploidy level of plants produced by the two methods showed the same range of variation, i.e., from haploid to tetraploid (Fig. 1). However, method 1 tended to produce more diploid and polyploid plants and less haploid plants (Table 4). This may be the result of simultaneous presence of cytokinin and auxin in the culture media, which has been shown to induce endoreduplication in cultured pea root cortex cells (Libbenga and Torrey, 1973). Endoreduplication has been observed in developing microspores of rice during anther culture by Chen and Wu (1983).

Table 4. Chromosome number of plants obtained from anther culture of rice

Culture method	No. of plants examined	Chromosome number			
		12	24	36	48
1	397	155 (39.0%)	194 (48.9%)	17 (4.3%)	31 (7.8%)
2	110	58 (52.7%)	47 (42.7%)	2 (1.8%)	3 (2.7%)

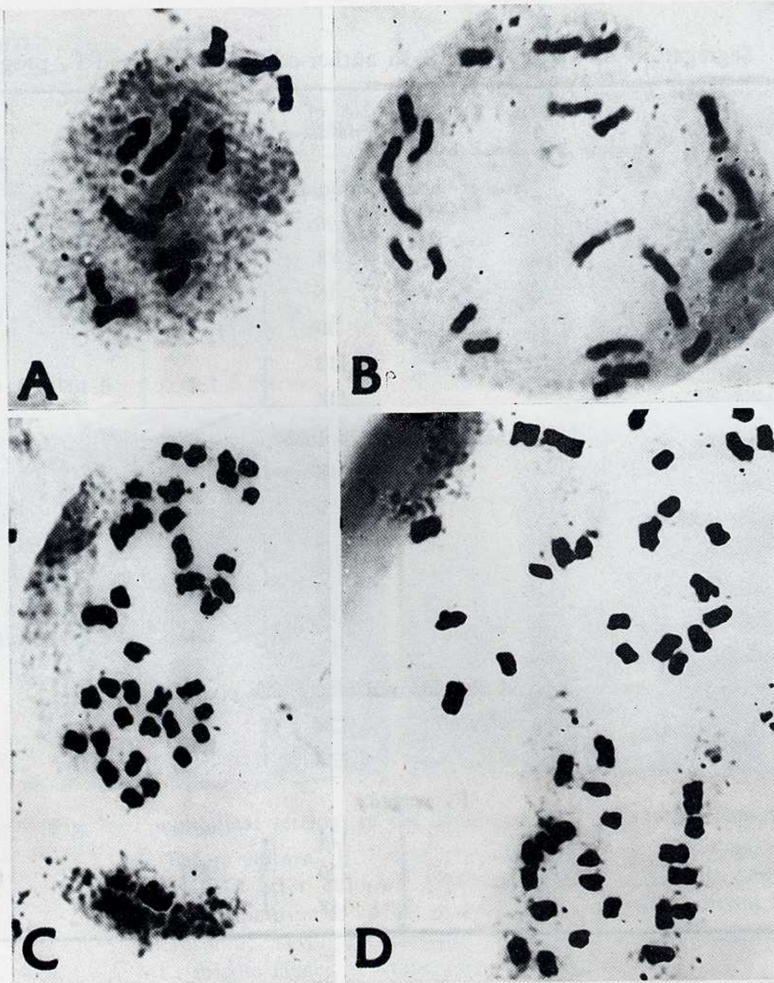

Fig. 1. The somatic chromosomes of anther-derived plants of rice.

A. Haploid showing 12 chromosomes. B. Diploid showing 24 chromosomes.
C. Triploid showing 36 chromosomes. D. Tetraploid showing 48 chromosomes.

Staining pollen grains with I_2–KI showed that only one type of pollen was present in the diploid and tetraploid plants produced by the two methods; simultaneous occurrence of nonwaxy and waxy pollen in a plant was not observed. Thus, all anther-derived diploid and tetraploid plants were homozygous. In both diploids and tetraploids the ratio of nonwaxy to waxy fits the expected 1:1 ratio, indicating that there has been no competition between the two classes of microspores during *in vitro* development. Normal segregation of the alleles of waxy gene was also detected in F_2 progeny (Table 5).

Histological observations revealed that the middle layer and tapetum of anthers at the mid-uninucleate microspore stage were already degenerating before culture was initiated (Fig. 2A). Response of anthers to the two callus-induction media was essentially similar. Degeneration of the middle layer and tapetum continued and by the 7th day of culture these two layers were no longer visible (Fig. 2B). Epidermal and endothecial cells remained quiscent throughout the culture. Limited cell divisions were observed in the filament and connective tissue during early period of the culture, but there was no indication that they

Table 5. Segregation of the waxy gene in anther-derived plants and F_2 progeny

Culture method	Genotype	No. of plants	Ratio tested	χ^2	P
	Anther-derived diploids				
1	wx^+/wx^+	96	1:1	0.266	0.70-0.50
	wx/wx	88			
2	wx^+/wx^+	16	1:1	3.674	0.10-0.05
	wx/wx	30			
Total	wx^+/wx^+	112	1:1	0.109	0.80-0.70
	wx/wx	118			
	Anther-derived tetraploids				
1	$wx^+/wx^+/wx^+/wx^+$	15	1:1	1.042	0.50-0.30
	$wx/wx/wx/wx$	9			
2	$wx^+/wx^+/wx^+/wx^+$	1	—	—	—
	$wx/wx/wx/wx$	2			
Total	$wx^+/wx^+/wx^+/wx^+$	16	1:1	0.592	0.50-0.30
	$wx/wx/wx/wx$	11			
	Anther-derived diploids and tetraploids pooled				
	wx^+/wx^+ & $wx^+/wx^+/wx^+/wx^+$	128	1:1	0	>0.95
	wx/wx & $wx/wx/wx/wx$	129			
	F_2 progeny				
	wx^+/wx^+	124	1:2:1	1.201	0.70-0.50
	wx^+/wx	220			
	wx/wx	118			

would form callus. On the other hand, a small proportion of microspores in the responsive anthers divided and formed multicellular units (Fig. 2C, D).

Thus genetic and histological data indicate that all anther-derived plants obtained by the two culture methods are of microspore origin; the haploid chromosome complement of some microspores must have multiplied spontaneously during *in vitro* development. The mechanism of genome multiplication during rice anther culture has been extensively investigated by Chen and coworkers. The cytological evidence presented by Chen and Wu (1983) for occurrence of nuclear fusion and endoreduplication in the developing microspores appears to be quite convincing. Endomitosis has also been inferred to occur both during early microscope development and subsequent proliferation of the callus (Chen and Chen, 1980), but its real existence has not been demonstrated.

Since the somatic tissues of rice anthers do not generally form callus on medium containing up to 2 mg/l 2, 4-D, other causes for occurrence of "heterozygosity" in anther-derived plants should be considered. Explanations frequently proposed are gene mutation (Oono, 1975; Burk and Matzinger, 1976; Burk *et al.*, 1979) after spontaneous chromosome doubling during *in vitro* microspore development and formation and subsequent development of microspores containing a 2*n* nucleus or two *n* nuclei (Sunderland, 1974; Wenzel *et al.*, 1976). As has been suggested by Chen and Li (1978), segregation for quantitative characters could also be the result of aneuploidy and changes in chromosome structure in anther-derived plants.

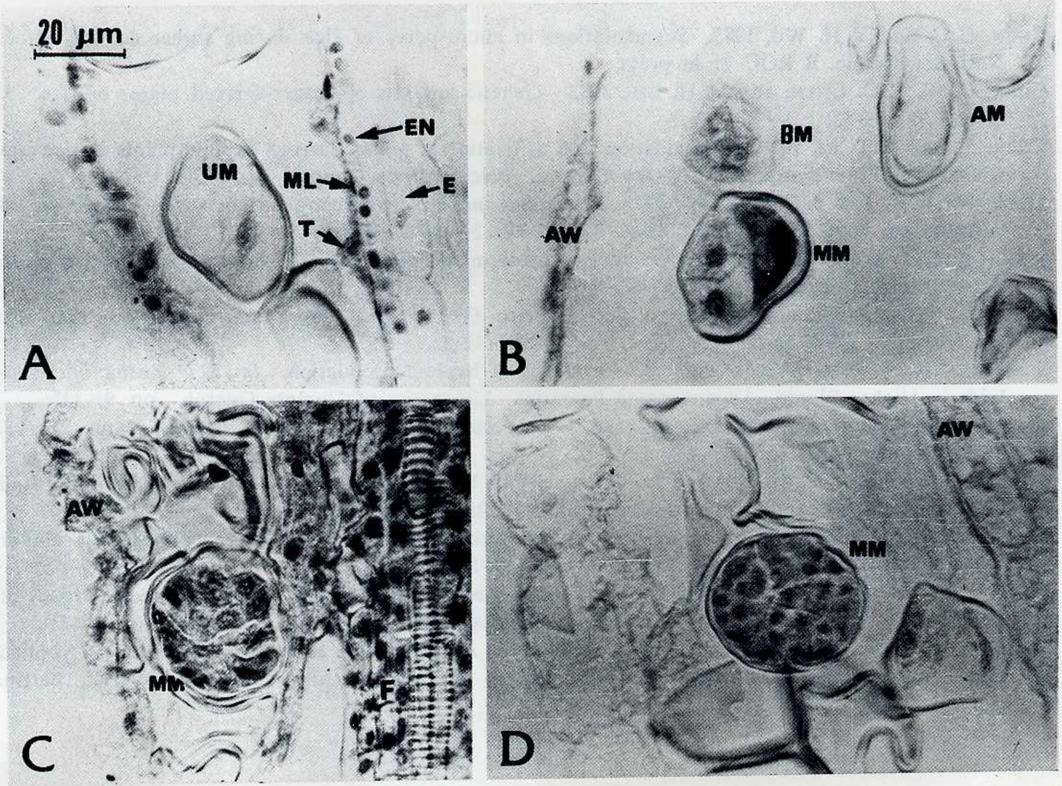

Fig. 2. Longitudinal sections of the anthers of rice before and after culture. A. Before culture. B. 7 days after culture. C. 14 days after culture. D. 21 days after culture. UM: uninucleate microspore; BM: binucleate microspore; MM: multicellular microspore; AM: aborted microspore; AW: anther wall; E: epidermis; EN: endothecium; ML: middle layer; T: tapetum; F: filament.

To understand the real causes of genetic variability in anther-derived plants, all these phenomena should be carefully studied.

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