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Expression of various gametic types in pollen plants regenerated from hybrids between *Triticum-Agropyron* and wheat

Z. Miao*, J. Zhuang and Hu Han
Institute of Genetics, Academia Sinica, Beijing, China

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Summary. Studies of the chromosomal composition of pollen plants regenerated from the F_1 of hybrids produced from *Triticum-Agropyron* intermediate type and common wheat demonstrated that various gametic types of the F_1 could be fully expressed at the whole plant level via anther culture. The observed frequency of each of the eight types of pollen plants (based on their chromosome numbers) was in good agreement with the theoretical probabilities as shown by X^2 analysis. Comparative studies of the chromosome composition of somatic cells and pollen mother cells (PMC's) of selected pollen plants permitted classification of the plants into four distinct classes. The majority of these regenerated pollen plants had identical chromosome numbers in both root tip cells and PMC's. An alien disomic addition line, which was cytologically stable for two generations, was obtained directly from anther culture. Moreover, the addition line exhibits resistance to stripe rust disease, a trait which is conferred by the *Agropyron* chromosome. We suggest that anther culture techniques provide a unique and expeditious route for the introduction of alien genes or chromosomes into wheat cultivars.

Key words: Anther culture – Intergeneric hybrid – Cytological characteristics – Gametic expression – Addition line

Introduction

It is well known that the use of haploids in plant breeding provides distinct advantages over the more traditional diploid hybridization approach. This is especially true for the rapid selection of mutant genotypes and in the acquisition of recessive genes which are usually concealed in heterozygous diploids. Haploid plants can be routinely produced by anther culture, and in recent years have been used successfully in the breeding of a number of agriculturally important crops (Li et al. 1984; Hu et al. 1984). Investigations on gametoclonal variation in the pollen plants regenerated from intergeneric hybrids (Wang 1984; Wang and Hu 1985) have demonstrated that anther culture is a powerful technique for chromosomal manipulation and could facilitate the utilization of desirable genes in wild species. A more thorough examination of the qualitative and quantitative expression of gametic types in haploid plants would not only increase our understanding of the mechanism(s) which lead to such selections in the culture regeneration process, but may also permit us to more effectively produce homozygous chromosomal variants through anther culture.

Triticum-Agropyron intermediate type (referred to hereafter as "intermediate type") is a genetically stable stock selected from later generations of a hybridization between *Agropyron intermedium* (Host P. B.) and common wheat (*Triticum aestivum* L.) (Hao and He 1979; Li and Sun 1981; Zhong and Mu 1983). This hybrid contains an entire complement of the wheat genome in addition to the genome of *Agropyron* (E genome). There are many desirable characters in the E genome still maintained in this intermediate type, such as immunity to wheat rust diseases (Wienhues 1966, 1973; Cauderon and Rhind 1976; Feldman and Sears 1981), immunity to streak mosaic disease, high protein content, drought resistance and cold tolerance (Anonymous Distant Hybridization Group of Heilongjiang Academy of Agricultural Sciences 1981; Yang 1982).

Pollen plants were first regenerated from anthers of *Triticum-Agropyron* intermediate type by Wang and Xu

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* Current address: Department of Biological Sciences, Rutgers University, Newark, NJ 07102, USA

(1975) who observed distinct morphological variations in a few pollen haploids (2 n = 28). Subsequent work by Zhuang and his colleagues on the anther culture of hybrids between common wheat and intermediate types increased our understanding of pollen plant regeneration, especially on factors which influence the induction frequency of green plants from these cultures (Zhuang and Jia 1981; Zhuang et al. 1985). However, there has been a paucity of research on the cytological aspects of these pollen-derived plants from distant hybrids.

In this report we describe a cytological survey of pollen plants regenerated from the distant hybrid between an octoploid intermediate type and common wheat and present evidence that all possible gametic types of the F_1 are fully expressed at the pollen plant level. We also demonstrate the potential of the anther culture technique as a vehicle for the efficient transfer of useful genes from wild species to wheat by the creation of an alien addition line.

Materials and methods

The plants used in this study were octoploid *Triticum-Agropyron* intermediate type No. 3 with a genome of AABBDDEE (2 n = 56), and hexaploid wheat (*Triticum aestivum* L. cv Orofen). Intermediate type No. 3 was crossed (as the female) with *Triticum aestivum* L. cv Orofen to produce a septuploid F_1. Anthers at the late uninucleate stage of the F_1 were inoculated on solid media. The culture method used was essentially that described by Zhuang et al. (1985). Potato-II medium (Chuang et al. 1978) was employed as the main induction medium, supplemented with 1.5 mg/l of 2,4-D and 0.5 mg/l of kinetin. Calli thus induced were transferred onto 190-2 medium (Zhuang et al. 1984) for plant regeneration, supplemented with 0.5 mg/l of naphthalene acetic acid and 0.5 mg/l of kinetin. The concentration of sucrose in this medium was 3% (w/v). Only plantlets regenerated from calli within two months were used in this study.

Root-tip chromosomes of pollen plants were examined at both the plantlet stage and when plants were transferred to pots in the greenhouse. Actively growing root-tips were excised under sterile conditions when they were approximately 3–4 cm long. Excised roots were pretreated at 0° to 1 °C for 24 to 30 h, fixed in 3 : 1 acetic–alcohol for 12–20 h, then stored at 4 °C until examination. Modified Carbol fuchsin squashing preparation was prepared as described by Wang (1984). After rinsing five times with tap water, root tips were macerated and incubated in a solution of 1% (w/v) cellulase and 1% (w/v) pectinase at 35 °C for 1 h, followed by additional fixing and squashing. At least 15 cells were counted for each plant, except for weakly growing plants where at least 10 cells were counted.

Pollen plants which were grown in the greenhouse were used for pollen mother cell (PMC) examination. Young spikes at the appropriate developmental stage were excised and fixed in 3 : 1 acetic–alcohol for 12 h, and the anthers were squashed in a modified Carbol fuchsin solution as described previously. Coverslips were removed following freezing in liquid nitrogen, and slides were air dried and mounted with Euparal.

Results

A total of 383 calli were induced from 11,510 anthers inoculated from the F_1 in an average induction frequency of 3.43%. Plantlets were regenerated in differentiation media as early as 5 days and continued for up to two months after transfer of the calli. Of the 256 plantlets regenerated in culture, 191 were green, accounting for 75% of the total plantlets obtained.

Variation of chromosome number in regenerated pollen plants

The root tips of 112 pollen plants (in pots) were examined for their chromosomal content. In this group we found 82 haploids (73.2%) and 30 diploids (26.8%), which presumably resulted from spontaneous doubling at some point in the regeneration process. In addition, there were two types of mixoploids observed. Mixoploid-A had chromosomal number variations at the same ploidy level within a plant; mixoploid-B had both haploid and diploid cells within the same plant. The former comprised 16%, and the latter 1.7%, of the total regenerated plants. As mixoploid-A type had no predominant number of chromosomes in the examined cells, they were excluded from the classification of pollen plants with regard to chromosome number. The results of these analyses are presented in Table 1.

The 92 pollen plants with a fixed chromosome number could be further classified into eight types according to their chromosome numbers, from $n = 21$ to $n = 28$ (or from 42 to 56 in diploids). In addition, a pollen plant was found that had cells which were predominantly $2n = 43$, which subsequently became a stable line with $2n = 42$ in H_2. No plants containing more than 56 chromosomes were observed. On a theoretical basis, one can predict the types of gametes produced with a defined number of chromosomes on the basis of random distribution of the seven univalents to either pole during meiosis. With this in mind, we performed a comparative study of the distribution of observed versus theoretically expected chromosomal classes in the regenerated pollen plants (Table 2). The results were subjected to a statistical analysis using the X^2 test which demonstrated no significant difference at 5% probability level. The fitness of the two distributions is clearly shown in Fig. 1. These results suggest that our experimental data match the theoretically predicted results in both a qualitative and a quantitative fashion.

PMC's in some pollen plants were examined for their chromosomal composition and compared with that found in somatic cells of the same plant (Table 3). It is significant that not all the plants examined had a consistent chromosome constitution in the two types of cells. Based on the uniformity and consistency of chromosomal composition in both of these cell types, the pollen plants could be divided into approximately four classes: those with (1) a uniform and consistent chromosomal composition in both cell types; (2) a generally consistent chromo-

Table 1. Number of chromosomes of pollen plants derived from F_1 between *Triticum-Agropyron* and common wheat

Chromosomes	*n*	21		22	23	24	25	26	27	28	A[a]	B[b]	Total
	2 n	42	43	44	46	48	50	52	54	56			
Haploids		0		3	14	16	19	10	6	2	12		
Diplods		1	1	3	7	6	4				6	2	
Total nos. of plants		1	1	6	21	22	23	10	6	2	18	2	112

[a] Mixoploids with chromosome variation at the same ploidy level within the same plant
[b] Mixoploids with both diploid and haploid cells within the same plant

Table 2. X^2* test of observed and expected plants with different chromosome numbers

Chromosome nos.	21	22	23	24	25	26	27	28	Total
Expected plants ($P = 1/2$)	0.72	5.0	15.1	25.2	25.2	15.1	5.0	0.72	92
Observed plants	2	6	21	22	23	10	6	2	92

* $X^2 = 8.021$; $P > 0.05$

Table 3. Comparison of chromosome constitution of both somatic cells and PMC's in some plants

Plant no.	Somatic cells			PMC'S			Classes
	Cells	Chromosome Constitution	%	Cells	Chromosome consitution	%	
177	17	22 + f[a]	88.2	113	22 + f	99.1	(1)
132	12	25	92.2	73	25	94.5	(1)
L-152	15	21	95.0	121	21	94.2	(1)
513	49	22	57.1	74	22	86.5	(2)
195	14	26	67.3	90	26	83.3	(2)
X-1	12	27	66.7	96	27	97.0	(2)
X-2	17	27	76.5	132	27	91.6	(2)
817	20	28	65.2	76	28	98.5	(2)
150	43	23	48.8	176	23	45.8	(3)
144	23	23	65.2	40	23	55.0	(3)
102	14	26	92.6	94	26	72.3	(3)
814-2	20	27	75.0	27	27	51.9	(3)
803	22	24 + f	63.5	54	23	90.6	(4)

[a] Chromosomal fragment

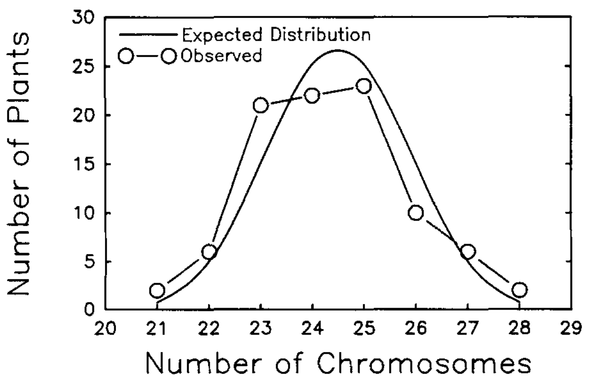

Fig. 1. Comparison between the distribution of observed pollen plants and that of theoretically expected plants

somal constitution but which is more uniform in PMC's than in somatic cells; (3) as above except that the somatic cells are more uniform than the PMC's; or (4) an obvious inconsistency between the two types of the cells. An example of this last category is found in plant 803, where 63.5% of the cells were $2n = 24 + f$ and 30% were $2n = 23$ in the somatic cells; in contrast, 90% of the PCM's examined (a total of 54) contained 23 chromosomes. Despite the inconsistencies described above, we have found that the great majority of regenerated pollen plants had a consistent chromosomal composition in both their somatic cells and PMC's. The phenomenon of chromosomal variation between root tip cells and PMC's, as seen in plant 803 and others, might be due to the fact that the plant originated from more than one cell (i.e. a chimera), or to chromosomal changes which occurred subsequent to shoot initiation (Karp and Maddock 1984).

Morphology and seed set of regenerated pollen plants

The doubled haploids regenerated from the hybrid did not perform as vigorously as normal diploids. However, the majority of doubled haploids did grow normally, with the exception of a few plants which grew slowly, produced few tillers and died before heading. Some doubled haploids did not set seeds after earring, partly due to suboptimal growing conditions. Many haploids had morphological aberrations and did not perform as well as normal diploids. A typical morphological variation seen in these haploids was ear branching and the abnormal elongation of the rachis segment length with sparse spikelets. The average rachis segment length in some variants was ten times as long as the *Triticum-Agropyron* parent. A similar variation was also observed in some of the doubled haploids. These morphological variations might result from the expression of recessive genes or from novel mutations.

The seed set of six plants which were examined cytologically in both the H_1 and the H_2 are shown in Table 4. Plant 228-1 was one of the typical alien addition line of wheat with a pair of 1E chromosomes which were marked with a satellite on the short arm (Fig. 2). This line is cytologically stable and morphologically uniform in both the H_1 and the H_2. Moreover, it was found to be resistant to strip rust disease, which makes it a potentially useful material for genetic studies and plant breeding. In addition, plant 225 has been converted into a stable line with 42 chromosomes in the H_2 from a mixoploid in the H_1 in which cells with 43 chromosomes were more predominant than that of 42 chromosomes. However, no morphological difference could be found between plant 228-1 and plant 225. Plant 582 was not very stable cytologically. Plant 220 and W-5 were observed to segregate in chromosome number in the H_2. Of five plants examined in the H_2 of W-5, three were $2n = 48$ and two $2n = 46$. Plant 205 still maintained $2n = 50$ in the H_2, although it did not perform normally. All of these lines are currently being continued for further investigations.

Table 4. Seeds set of doubled haploids

Plants	H_1		H_2	
	Chromosome nos.	Seeds set	Chromosome nos.	Seeds set rate (seeds/spikelets)
220	41 + t[a]	27	41, 41 + t, 42	1.5
225	43	13	42	1.6
228-1	44	14	44	1.8
582	46–50	8	50	1.7
w-5	48	12	46, 48	0.96
205	50	5	50	1.3

[a] Chromosomal telosome

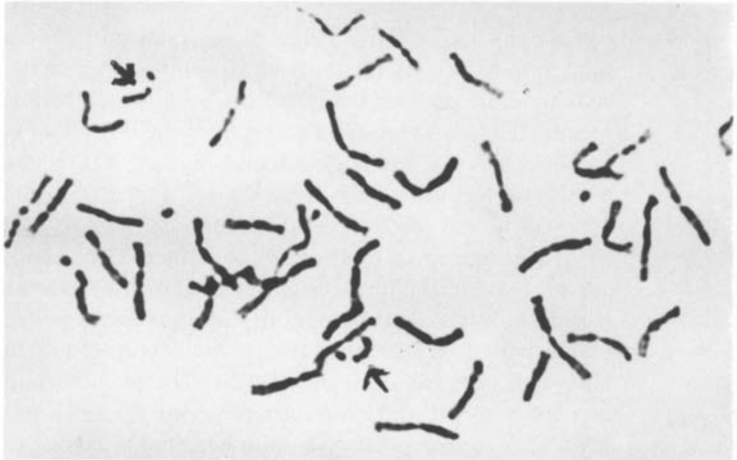

Fig. 2. The chromosome constitution of pollen plant 228-1 (H_2) of common wheat with a pair of 1E *Agropyron* chromosomes marked with a satellite on short arm indicated by the *arrow*

Fig. 3. The spikes of pollen-regenerated plants derived from *Triticum-Agropyron* intermediate type No. 3 crossed with *Triticum aestivum* L. cv Orofen and their parents from *left to right*: intermediate type No. 3 (♀), plant 205 with 50 chromosomes, plant 228-1 with 44 chromosomes, plant 225 with 42 chromosomes, and Orofen (♂)

Discussion

By the use of anther culture, we have regenerated 191 green pollen plants from the F_1 of a hybrid between intermediate type and hexaploid common wheat. Cytological examination showed that all eight types of plants, as theoretically predicted with chromosomes ranging from $n = 21$ to $n = 28$, were obtained. This suggests that anther culture is an expeditious route for the expression of the various types of gametes at the whole plant level. Most of the regenerated pollen plants had a constant chromosomal composition in both somatic cells and pollen mother cells. A homozygous alien addition line and several other chromosomal variants were directly acquired via anther culture, which greatly reduced the time normally required for the production of such lines by traditional hybridization techniques.

Expression of various gametic types at the whole plant level via anther culture

The creation of desirable gametes which will be expressed at the whole plant level is one of the most crucial steps in plant breeding. The realization of gametic expression in conventional breeding requires a process of fertilization in which, to obtain the desired homozygous diploid individual, there must be correct fusion of two identical or correspondingly similar gametes from both the male and female gametophytes. Due to a large number of permutations possible, this usually requires a large population of parents for selection in breeding programs and the screening of a large number of progeny for the correct gametic combination. This process is, therefore, extremely time consuming and labor intensive. In addition, agronomically desirable traits are often recessive and normally concealed by dominant alleles. This is especially true for quantitative recessive genes which are quite difficult to get properly recombined in heterozygous diploids. The induction of haploids through anther culture with subsequent chromosome doubling should allow one to circumvent these problems. One of the most surprising, and most useful, findings of our study is that the various types of gametes that one expects to be formed in the parents could randomly develop into pollen plants.

Additionally, we are unaware of other reports of gametic expression at the pollen plant level. The fact that this phenomenon has not been reported previously, however, may be due to the use of an experimental design. Wang and Hu (1985) pioneered the area of plant chromosomal manipulation through anther culture by employing hexaploid triticale and common wheat to produce the mixoploid hybrids for anther culture. They found significant variations, both cytologically and morphologically, in the regenerated pollen plants but their analysis was complicated by an aberrant segregation of chromosomes at meiosis in the mixed genome. In contrast, we have avoided such inherent complications by employing a septuploid hybrid with a single genome in a univalent state. The gametes formed in such a septuploid would have a range of chromosome numbers from 21 to 28, as each of the seven univalents in the E genom distributes randomly to either pole during meiosis. Hence, the transmission rate of each univalent into any one gamete should be 50%. Therefore, the probability for each kind of gamete can be calculated by the following binomial formula:

$$F(x) = \frac{7!}{x!\,(7-x)!} \cdot \left(\frac{1}{2}\right)^7$$

where F(x) signifies the probability of any gamete with “x” E chromosomes. The unique feature of this experimental design is that it is easy to compare the observed distribution of chromosomal consistency in different kinds of pollen plants with those of the expected. We have shown that the two distributions yielded an excellent fit without any statistically significant difference. It is noteworthy, however, that the observed curve shifts slightly towards the left (to the side with plants containing fewer chromosomes) although the overall shapes of the two curves are similar. It appears that the gametes with chromosomes closer to the chromosome number of the intact genome have a slight advantage in subsequent development into whole plants. The only exception is the gametes with 23 chromosomes, where the observed pollen plants (21) are more than those of expected (15.1). A similar phenomenon was reported by Wang and Hu (1985). The fact that gametes with 23 chromosomes are favored for pollen plant regeneration suggests that these gametes may somehow be more competitive than other aneuploid gametes, and, therefore pollen plants with 46 chromosomes are more frequently produced and stabilized through anther culture.

Rapid creation of homozygotes with alien chromosomes

In this study, an alien disomic addition line was produced directly through anther culture. This line is cytologically stable, with normal seed set, and is resistant to strip rust disease. This line has gone through two generations and may prove to be agronomically valuable for wheat breeding and future genetic research. The significance of this result lies not only in the production of a specific disomic addition line per se, but in the generalized application of this approach for the rapid production of various homozygotes harboring alien genes. Although modern breeding methods have produced tremendous advances in crop fitness and yield, the lack of gene resources within cultivated plants has sorely limited further development. To date, the most practical approach to the utilization of wild germ resources is by means of chromosomal manipulation. The incorporation of alien chromosomes or specific genes into wheat generally necessitates the use of addition lines, substitution lines, or translocation lines. These techniques have been successfully exploited by a number of researchers for several decades through conventional hybridization between wheat and its distant relatives (Hyde 1953; Dvorak and Knott 1974; Islam et al. 1981). One major drawback with conventional methods, however, is that it usually takes from three to five generations to isolate disomic addition lines from hybrids of the amphiploid and wheat. Likewise, acquiring substitution lines requires at least another four generations by utilizing addition lines and the corresponding aneuploids of wheat (Feldman and Sears 1981; Miller 1984). In contrast, addition lines and substitution lines can be directly obtained through anther culture of appropriate materials. The haploid approach has the advantage of not only greatly reducing the time required to establish such lines, but also has a much higher efficiency in the production of homozygous variants (Islam et al. 1978, 1981).

The selfing of monosomic or double monosomic addition lines is currently the only way to develop disomic addition lines in conventional breeding programs. This approach suffers from the fact that the transmission rate of male gametes with alien chromosomes is extremely low and, thus, wheat pollen with an extra chromosome(s) is much less likely to achieve fertilization than normal pollen. By comparison, the use of haploids completely circumvents this problem. By calculation, the frequency of alien disomic addition lines which occur in the F_2 of octoploid intermediate type and wheat is about 0.3%, whereas we have found frequencies 18 times higher through the anther culture of the F_1. The observed frequency of regenerated pollen plants with 22 or 44 chromosomes was 5.36%, very close to the expected value of 5.34%. In this study, all of the diploids were doubled spontaneously, without artificially doubling. In addition, some novel genetic forms which are very difficult to acquire by self-fertilization, e.g. plants with 46 or 48 chromosomes, might be developed through anther culture. Even though these types may occur at a low frequency in the offspring of self-hybrids, most would be expected to be heterogenous, and the genotypes of the desired gamete may be readily lost or altered in subsequent generations (G. Zhong, personal communication). The alternative (and preferred) approach would be to produce honozygotes by the doubling of pollen-derived haploids. In this study we have produced doubled haploids with both 48 and 50 chromosomes. Although the W-5 isolate segregated in chromosome number in the H_2, the segregation was obviously different from that of heterogenous plants where one sees a much broader range of segregation. We would like to suggest that the approach described in this paper may be suitable for the rapid production of novel wheat genotypes, such as plants with more than 44 chromosomes, and that such plants may have tremendous value to both the applied and basic aspects of genetic research.

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