Comparative study of symmetric and asymmetric somatic hybridization between common wheat and *Haynaldia villosa*

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Abstract Symmetric and asymmetric protoplast fusion between long term cell suspension-derived protoplasts of *Triticum aestivum* (cv. Jinan 177) and protoplasts of *Haynaldia villosa* prepared from one-year-old embryogeneric calli was performed by PEG method. In asymmetric fusion, donor calli were treated with gamma ray at a dose of 40, 60, 80 Gy (1.3 Gy/min) respectively and then used to isolate protoplasts. Results of morphological, cytological, biochemical (isozyme) and 5S rDNA spacer sequence analysis revealed that we obtained somatic hybrid lines at high frequency from both symmetric and asymmetric fusion. Hybrid plants were recovered from symmetric and low dose γ-fusion combinations. GISH (genomic *in situ* hybridization) analysis proved exactly the existence of both parental chromosomes and the common occurrence of several kinds of translocation between them in the hybrid clones regenerated from symmetric and asymmetric fusion. And the elimination of donor DNA in hybrid clones regenerated from asymmetric fusion combinations was found to increase with the increasing gamma doses. It is concluded that transference and recombination of nuclear DNA can be achieved effectively by symmetric and asymmetric fusion, hybrids with small fragment translocation which are valuable in plant breeding can be obtained directly by asymmetric fusion.

Keywords: common wheat, *Haynaldia villosa*, somatic hybridization, genomic *in situ* hybridization.

Common wheat (*Triticum aestivum*, 2*n* = 42, AABBDD) is one of the most important cereal crops in the world. The enhancement of its yield, quality and tolerance to various stresses is significant for the livelihood of the people. In its wild closely related plants there exist many genes desired for its improvement. Introgression of such genes into wheat is expected to be an important way to enrich the germplasms of wheat. *Haynaldia villosa* (2*n* = 14, VV), an annual grass originated from the shore of Mediterranean sea, is characterized with strong tillering power, abundance in spikelets (ears), resistance to rust and take-all fungus, immunization against powdery mildew and high seed protein and lysine content. Therefore it is a good resource for wheat improvement. Sexual intergeneric hybridization has already been performed in the 1930s by some investigators and some alien additional and substituted lines had been created. However, compared with *Secale*, *Elytrigia* and *Aegilops*, successful utilization of *H. villosa* in wheat breeding is much fewer. Sexual cross between common wheat *and H. villosa* is difficult because it requires long duration for many terms of backcross before it can be used for breeding. Somatic hybridization breaks a new

path for plant breeding, especially asymmetric somatic hybridization, which was raised at the end of the 1980s, is superior to symmetric for it produces hybrids possessing comparatively fewer chromosomes (genes) from donor, thus avoiding too much wild traits introduced into the acceptor and thus the traits of the hybrids were nearer to the goal of breeding.

This work carried out symmetric and γ-fusion between common wheat and *H. villosa* simultaneously in order to investigate the influence of different irradiation dosages upon the chromosome elimination as well as the growth and development of the hybrids. The behaviors of alien chromosomes in hybrids of symmetric and asymmetric fusion were also investigated.

1 Materials and methods

1.1 Plant material

Protoplasts of common wheat cv. Jinan 177 were prepared from cell suspensions which were established from light yellow fragile calli[1]. The regeneration capacity of protoplasts was almost lost because of long-term culture. While they can divide with high frequency.

Donor protoplasts of *H. villosa* derived from embryogenic calli, which have been subcultured for one year[2]. Color of the calli was lighter than that of wheat. The calli were composed of 1.5 mm granules. Regeneration capacity of the calli decreased rapidly during the subculture and was very low when they were used as the fusion partner in the fusion experiments.

1.2 Isolation of wheat protoplasts

Protoplasts of wheat were prepared from cell suspensions after 2—4 days of subculture according to Zhou et al.[2].

1.3 ^{60}Co-γ ray irradiation and the preparation of *H. villosa* protoplasts

After 4 days of subculture, calli of *H. villosa* were treated with ^{60}Co-γ ray irradiation at the doses of 40, 60 and 80 Gy respectively with a dose rate of 1.3 Gy/min (cf. ref. [3]). On the same day or 2nd day, calli were picked out to prepare protoplasts with the above-mentioned method for wheat protoplasts isolation.

1.4 Protoplast fusion and culture

Protoplasts of both parental were mixed at 1∶1 ratio and then fusion experiments were carried out by PEG method[5]. There were four fusion combinations:

A: wheat protoplasts (+) untreated *H. villosa* protoplasts

B: wheat protoplasts (+) *H. villosa* protoplasts prepared from irradiated calli at a dose of 40 Gy (1.3 Gy/min)

C: wheat protoplasts (+) *H. villosa* protoplasts prepared from irradiated calli at a dose of 60 Gy (1.3 Gy/min)

D: wheat protoplasts (+) *H. villosa* protoplasts prepared from irradiated calli at a dose of 80 Gy (1.3 Gy/min)

Among them, A is symmetric fusion combination and B—D are asymmetric fusion combination. Cultures of each parental untreated protoplasts and their mixture were used as controls. One to two experiments (at least 4 repeats per experiment) per fusion combination were performed.

When the regenerated calli or somatic embryos grew to 1.5—1 mm or more in diameter, they were picked out respectively and cultured on the proliferation medium[4]. After being subcultured for 1—2 terms, the proliferated calli were transferred to the differentiation medium[5]. Regenerated seedlings which were 5 cm or more in height were transferred to seedling strengthening medium[5]. The seedlings were potted into soil after they had transported and survived in the paper cup.

1.5 Identification of regenerated calli and plants

1.5.1 Morphological, cytological and isozyme analysis. Color and size of the granules of the regenerated calli were observed and compared with that of both fusion partners, and these data were used as evidence of their hybridity. So did the vegetative and reproductive organs of the regenerated plants such as epidermal hairs, color of the stem and shape of flowers.

Chromosome preparations were obtained according to the method of Xia et al.[5]. After being stained with 5% Giemsa, the chromosome slides were observed under microscope.

Isozyme analysis was performed for esterase. Samples were extracted from regenerated calli and leaves of regenerated plants. Extraction and procedure of polyacrylamide gel electrophoresis were the same as Xia et al.[5].

1.5.2 Molecular analysis. (i) Genomic *in situ* hybridization (GISH). Chromosome plates of regenerated calli were prepared with methods of squashing or dropping as described by Xia et al.[5]. Good samples were used after air-dried for 1—2 days or stored at –20℃.

Preparation of probe: Total genome DNA of *H. villosa* were extracted by CTAB method[6] and labeled using nick translation kit following the manufactures' instruction (976776, Boehringer Mannheim). The hybridization mixture (18 μL per slide) consisted of 50% formamide, 10% dextra sulfate, 2 × SSC, 0.5 μg/mL ss DNA, 20 ng of *H. villosa* probe DNA and 2 μg blocking DNA (total genome DNA of wheat cv. Chinese spring). After being hybridized in humid box at 37℃ overnight, the slides were washed and detected following the procedure of Fluorescent Antibody Enhancer Set for DIG Detection kit (1768506, Boehringer Mannheim). The slides were stained with 4 μg/mL propidium iodode (PI), mounted with anti-fate (Voctashield, Vector laboratories, CA94010). Fluorescent images were observed under a fluorescence microscope (Olympus BX60).

(ii) 5S rDNA spacer sequence analysis. Total genome DNA of regenerated calli and plants were prepared by CTAB method. A pair of 25mer primers was used to amplify the 5S rDNA spacer. Sequence of the primers and PCR procedure were the same as ref. [7].

2 Results

2.1 Culture of fusion products and regeneration of plants

The development of fusion products was different between the 4 fusion combinations we designed (table 1). Comparing with the asymmetric fusion, the number and growing speed of regenerated cell clusters from symmetric fusion were more and faster. Dense embryogeneric calli were formed after 1 month of culture. Among the asymmetric fusion combinations, the number of regenerated calli from combination C (60 Gy) was the most and there was nearly no difference between combination B (40 Gy) and combination D (80 Gy). After 2 months of culture, the early-formed calli (2 mm in diameter) were picked out and transferred to proliferation medium simultaneously. Behavior of calli derived from different fusion combination was different when transferred to differentiating medium. Differentiation of plants was earlier in symmetric fusion combination than asymmetric fusion combinations. Only after several days on the differentiation medium, calli regenerated from symmetric fusion combination A started to differentiate. The period from fusion to the formation of whole green plants was only 3 months. Its remarkable characteristic was that most plants regenerated via somatic embryo pathway. More than 90% of the regenerated plants were originated from this pathway in the early 2 months. Thereafter regenerated plants were formed mainly from organgenesis and the differentiation of roots was more difficult. According to the statistics, among the 56 regenerated clones in symmetric fusion combination A, many whole green plants were regenerated and survived from 8 clones (8/56=14.3%); green spots, roots or albinos were regenerated from 30 clones, i.e. 53.6% (30/56) of them had low regeneration capacity; 21 clones were non-regenerable (21/56=32.1%). Only one clone from asymmetric fusion combination B (40 Gy) and C (60 Gy) gave whole green plants respectively 2 months later than symmetric fusion. Regeneration frequencies of fusion combination B and C were 2.8% (1/36) and 2.5% (1/40) respectively. About 25% of the regenerated clones had low regeneration capacity. No whole plants regenerated from combination D (80 Gy), only green spots or albinos were recovered from 10% of regenerated clones after 1 year's culture.

Table 1 Development of parental protoplasts and fusion products

	Time when the first division occurred/d	No. of cell clusters after 15 days' culture	State of regenerated calli after 40 days' culture		Differentiation of regenerated calli (clones which could regenerate whole green plants/clones which were transferred onto the differentiation medium)
			size/mm	number	
Protoplasts of wheat (recipient)	2—3	—	—	—	—
Protoplasts of *H. villosa* (donor)	—	—	—	—	—
Fusion combination A	3 — 4	++++	1	++++	8/56
Fusion combination B	4 — 5	+++	0.5—1	+++	1/36
Fusion combination C	4 — 5	++++	0.5—1	+++	1/40
Fusion combination D	6 — 8	+++	0.5—1	++	0/40

As the control, wheat protoplasts could not grow further after several dividing cycles. No calli were obtained. Protoplasts of *H. villosa* could not divide under the conditions of this experiment. Mixture of both parental protoplasts divided on the 3rd to 4th d of culture, formation and the growing speed of cell clusters was slower than that of symmetric fusion combination, number of regenerated calli was less than all the fusion combinations and no regenerated plants were obtained.

2.2 Hybrid nature identity of the regenerated calli

2.2.1 Morphological, cytological and isozyme analysis of the regenerated calli. Calli of common wheat were fragile and composed of light yellow small granules (T type), while the calli of *H. villosa* were dense and consisted of pale yellow bigger granules (H type). All the regenerated calli from symmetric fusion A were middle type (M type); calli regenerated from γ-fusion were either M type or T type. Table 2 showed the ratio of different types.

Table 2 Morphological, cytological and isozyme analysis of fusion products

Fusion combination	Morphology of regenerated calli		Main range of chromosome number	Pattern of eaterase			Result of 5S rDNA spacer sequence analysis	
	M(%)	T(%)		P.N(%)	P(%)	T(%)	P(%)	T(%)
A	100.0	0	30—45	0	100.0	0	71.4	28.6
B	67.0	33.0	24—40	16.7	50.0	33.3	33.3	66.7
C	58.2	42.8	25—38	33.3	66.7	0	42.1	47.9
D	45.6	44.4	20—40, with CF	100.0	0	0	5.8	64.2

M, Middle type; P, having characteristic band(s) of both parents; N, having new band(s); T, having characteristic band(s) of common wheat; CF, chromosome fragment(s).

Chromosome number of common wheat ($2n$= 42) decreased remarkably during the long-term culture. There were only 24—28 chromosomes in most cells when they were used as the fusion partner. Chromosome number of *H. villosa* also changed during one-year's culture. The main range is 11—14. Chromosome numbers of calli regenerated from symmetric fusion were near to the sum of both parents, those of calli regenerated from asymmetric fusion were less and different with the irradiation dose. Chromosome numbers of calli regenerated from three asymmetric fusion combinations (40, 60, 80 Gy) were mainly 24—40, 25—38 and 20—40 respectively. Chromosome fragments were found in high-dose fusion combination D (80 Gy) (table 2).

All of the clones analyzed by esterase isozyme had all or part of the characteristic band(s) of both parents (P type); clones regenerated from γ-fusion had part of the characteristic band(s) of wheat (P) as well as new band(s) (T. N type) , very few clones were T type. Probability of appearance of new band(s) increased with the rising of the irradiation dose (table 2). The existence of new band(s) demonstrated the recombination of parental genetic materials in the regenerated clones.

Results of 5S rDNA spacer sequence analysis showed that most clones regenerated from symmetric fusion had characteristic band(s) of both parents, a few clones did not have the characteristic band(s) of *H. villosa* (table 2). On the contrary, only a few clones regenerated from asym-

metric fusion had characteristic band(s) of both parents (table 2). This result revealed that γ-ray irradiation caused the missing of 5S rDNA coding sequence in the hybrids from the level of gene loci.

2.2.2 GISH analysis. Results of GISH (table 3) showed that chromosome numbers of wheat and *H. villosa* were usually 23—28 and 13—14 respectively in cells of calli regenerated from symmetric fusion combination. Doubling of chromosomes and 1—2 recombined chromosomes were also found in some of the clones (fig. 1(a)). While in calli regenerated from γ-fusion combinations chromosome numbers of wheat were usually 21—28 and intact chromosome from donor *H. villosa* decreased with increasing irradiation dose. In the three γ-fusion combinations (40, 60 and 80 Gy), chromosomes of *H. villosa* and recombinant in the cells were 9—12/0—4, 7—8/2—3 and 0/5—8 respectively (fig. 1(b), (c), (d)). No whole *H. villosa* chromosomes were found in the cell of calli derived from high-dose γ-fusion combination D (80 Gy) (fig. 1(c)), but hybridization signal appeared near the centramere and telomere regions. Difference of chromosome behavior in the hybrid cells between symmetric and asymmetric fusion combinations was that in clones of asymmetric fusion small fragment translocation appeared (fig. 1(b), (c), (d)). According to the results of GISH, there were both parental chromosomes and recombined chromosomes in the hybrid clones regenerated from both symmetric and asymmetric fusion.

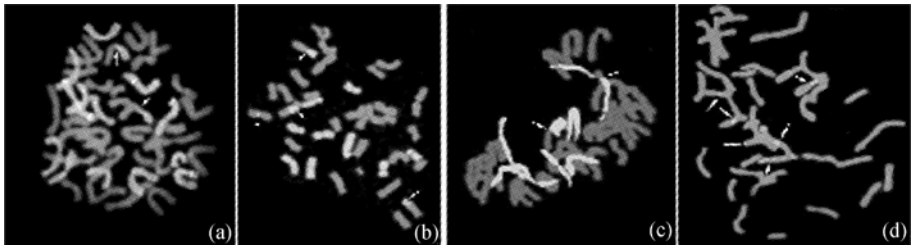

Fig. 1. GISH results on clones regenerated from symmetric and asymmetric fusion. (a) GISH result on clone No. 27 regenerated from symmetric fusion combination A. ↑: recombined chromosome; (b) GISH result on clone No.5 regenerated from γ-fusion combination. B (40Gy). ↑: recombined chromosome; (c) GISH result on clone No. 1 regenerated from γ-fusion combination C.(60Gy). ↑: recombined chromosome; (d) GISH result on clone No. 12 regenerated from γ-fusion combination D (80Gy). ↑: recombined chromosome.

The above results from morphological, cytological, biochemical (isozyme), GISH and 5S rDNA spacer sequence analysis revealed that all the clones regenerated from symmetric and γ-fusion we analyzed were somatic hybrids.

2.3 Hybrid identification of regenerated plants

The hybrid nature of regenerated plants was verified by morphological, isozyme and 5S rDNA spacer sequence analysis.

The gross morphology of the plants regenerated from symmetric fusion resembled that of wheat. However, epidermal hairs of most plants were long, thick and scattered like that of *H. villosa,* while those of wheat were short, thin and dense. The stems of some plants were violet.

Whole plants regenerated from asymmetric fusion resembled the morphology of wheat.

Table 3 Results of GISH on regenerated calli

Fusion combination	No. of clones	Chr. number of the cell	Chr. number of wheat in the cell	Chr. number of *H. villosa* in the cell	Number of recombined chromosome in the cell
A	1	38 — 45	24 — 28	13 — 17	0 — 2
	10	40	26	14	0
	13	76	48	28	0
	14	52+2CF	24	25	3+2CF
	19	29 — 42	20 — 28	8 — 14	0 — 2
	27	38 — 43	23 — 28	13 — 14	1 — 2
B	5	37 — 38	21 — 26	9 — 12	3 — 4
	11	31 — 39	21 — 28	7 — 11	0 — 4
C	1	30 — 38	20 — 27	7 — 8	2 — 3
D	12	23 — 39	23 — 39	0	6 — 7

F, chromosome fragment; chr, chromosome.

All the analyzed plants regenerated from symmetric fusion (9-1, 9-5, 10-2, 13-4 and 27-1) and asymmetric fusion (γ-fusion combination C (60 Gy): 1-4) had characteristic bands of both parents in esterase pattern. Among them No. 9-5 and 13-4 plants regenerated from symmetric fusion and No. 1-4 plant regenerated from asymmetric fusion had new band(s), which demonstrated that genetical materials of both parents existed in plants regenerated from symmetric and asymmetric fusion combination and genetical recombination also existed in some plants.

In electrophoresis pattern of PCR products of 5S rDNA spacer sequence analysis, all the analyzed plants regenerated from symmetric fusion (13-3, 18-1, 18-2 and 27-1) had characteristic bands of both parents. No.1-1 plant regenerated from asymmetric fusion combination C (60 Gy) also had characteristic band(s) of both parents, while No.1-3 plant regenerated from the same asymmetric fusion combination had not the characteristic band of *H. villosa*.

2.4 Development of regenerated hybrid plants

Regeneration of plants from symmetric fusion was earlier and the number of plants was more than that from asymmetric fusion in the same fusion experiment. Viability of plants regenerated from different fusion combination was different. Hybrid plants regenerated from symmetric fusion were healthy and strong, while those from asymmetric fusion were weak. Only No.11 clone gave tall and slender seedlings from 40 Gy γ-fusion combination B. From 60 Gy γ-fusion combination C, No.1 clone gave several plants, but leaves of the plants were light green and crimpled (fig. 2) and some of them with white stripes or albinos.

Fig. 2. Weak plants regenerated from 60Gy γ- fusion combination C.

Plants regenerated from asymmetric fusion could not grow further after being potted in soil.

Somatic hybrid plants regenerated from symmetric fusion grew to maturation outdoors. The appearance of spikes resembled that of wheat (fig. 3(a), (b)), whereas the ovaries and anthers were intermediate between two parents (fig. 3(c)). Only one hybrid plant regenerated from No. 13 clone set seed. The other plants were sterile. In order to rescue the generation, their ovaries were induced to reform many plantlets which grow well in soil.

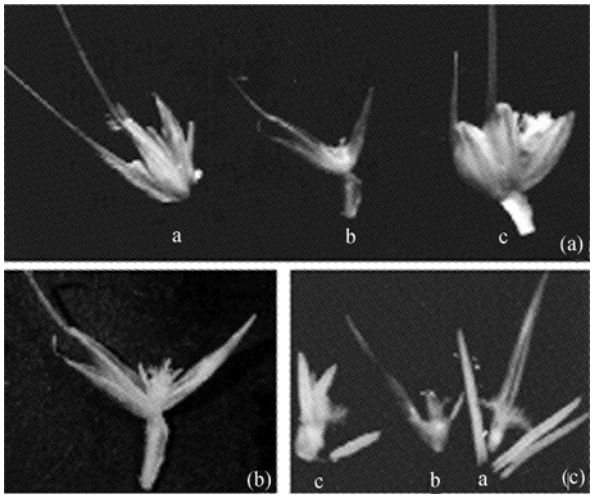

Fig. 3. Comparison of flower between somatic hybrid and parents. (a) Comparison of flower appearance: a, *Haynaldia villosa*; b, hybrid plants; c, wheat. (b) Flower of hybrid plant. (c) Comparison of ovary and anthers: a, *Haynaldia villosa*; b, hybrid plants; c, wheat.

Put all together, in the case of wheat lacking considerable chromosomes (with chromosome nos. 24—28), symmetric and asymmetric hybridization between it and *H. villosa* was still capable of producing hybrid plants due to complementary effect of regeneration and those from symmetric fusion were healthy and normal and grew to flowering.

3 Discussion

3.1 Analysis of chromosome behavior in hybrids by means of genomic *in situ* hybridization (GISH)

To prove the introgression of alien gene(s) into wheat via cell fusion, the verification of the presence of alien chromosomes is very important. Although morphological, cytological and biochemical assays are commonly used, GISH has become a preferable technique to inspect hybrid chromosomes for its exactness and direct visualization in results since the 1990s[8,9]. In symmetric and asymmetric hybrids, translocations involving alien chromosomes or genomes are commonly observed by this technique[10—13]. However no work has been done to analyze the influence of irradiation dosage on chromosomes by GISH.

Via GISH technique, the common occurrence of translocation of alien chromosomes is also proved in this experiment. High frequency (80%) of this translocation was observed in the examined cell lines of hybrids, irrespective asymmetric or symmetric ones. Besides, we have investigated by means of GISH for the first time the influence of different dosages of γ-ray in the fusion between wheat and *Haynaldia villosa*. The experimental results showed that the increase in dosage induced not merely the greater extent of chromosome elimination, but also the occurrence of recombination of chromosomes at multiple loci. In combination D (irradiation dosage was 80 Gy) there were only scattered hybridization signals in the hybrid cells of regenerated calli. This result

was alike in several repetitions while seldom such signals were present in other combinations with low dosage of irradiation. The fact that small amount of alien chromosomes in the form of mini fragments was inserted into the acceptor chromosomes is interesting, for the alien genes can be kept stable there. Besides it is advantageous to the marking of the desirable alien genes and their cloning. This result is of significance both in theory and breeding practice. Although no normal regenerated plants were obtained from the fusion combination D (80 Gy), it is expected that regenerated plants and progenies carrying translocational (inserted) mini alien chromosomes would be gained through the following experiments when cultured cell system of wheat with comparatively complete set of genome is established and used in fusion.

The result of GISH showed that regenerated clones from symmetric fusion possessed the whole genome of the donor *Haynaldia* while in that of the γ-fusion, the chromosome number of *H. villosa* decreased as the irradiation dosage was raised, and their recombination frequency increased. This is in correspondence with the results of the esterase isozyme and 5S rDNA space sequence analysis, that is the probability of the presence of new bands in esterase isozyme pattern increased, the numbers of characteristic bands of *Haynaldia villosa* decreased and the probability of loss of 5S rDNA gene coding sequences from *Haynaldia villosa* increased along with the enhancement of irradiation dosage.

3.2 Better growth of hybrid and complementation of regeneration capacity between both parents in somatic hybridization of wheat

Shieder et al.[14] has observed for the first time the better growth rate of hybrid calli than those of the parents in somatic hybridization between *Datura* species and considered this phenomenon as the result of a heterosis effect and proposed utilizing it in the isolation of somatic hybrids. This strategy has been successfully employed in some instances especially potato[15—17]. In the present work, more than 100 regenerated clones early formed were identified as hybrids, which proved the existence of this effect from cytological, biochemical and molecular level.

There were rare reports in the past about the regeneration of whole plants via fusion between both parents with low or even none totipotency. This phenomenon was observed in the symmetric fusion between wheat and *Agropyron elongatum*[4], γ-fusion between wheat and *Haynaldia villosa*[2] as well as UV fusion between wheat and *A. elongatum*[18], also wheat and *Psathyrostachys juncea*[18]. Because of its constant presence, it may be utilized in the selection of fusion products. Together with the better growth effect of hybrid, they provided a reliable method for hybrid selection of wheat without any treatment such as IOA upon the acceptor also occurring in this experiment.

It is noticeable that according to the results of chromosome counting and GISH, most of the hybrid clones irrespectively derived from symmetric or asymmetric fusion, which were capable of regenerating to whole plants, always contained nearly 42 chromosomes (the normal chromosome numbers of wheat) from both parents. Those possessing much more or less chromosomes were

low and none regenerable (except a few double diploids, which could regenerate normal plants). In the present experiment, acceptor wheat had lost considerable amount of chromosomes ($2n$ = 24—28) and only when they were supplemented by enough alien donor chromosomes from symmetric fusion could the hybrid clones differentiate to healthy and normal plants at higher frequencies. But in asymmetric fusion as the donor chromosomes had been eliminated by irradiation, less chromosomes existed in hybrid clones, which produced only weak and abnormal seedlings at lower frequency or even non-regenerable at high doses of irradiation. In other analogue experiment between wheat just with 30—38 chromosomes and *H. villosa*[2], the hybrid clones formed from γ-fusion had 40—48 chromosomes and healthy plant with high frequency.

Based on the above comparison and from the viewpoint of plant regeneration, the strategy for the somatic hybridization of wheat can be considered as follows: In the case of wheat cultures having comparatively complete chromosome sets, asymmetric fusion should be adopted for producing hybrid plants with traits more like wheat. Contrarily when more chromosomes has been lost in wheat cultures, symmetric fusion is preferable.

The complementary effect is also influenced by varieties of wheat, no plant regenerated from the symmetric and asymmetric fusion of other genotype wheat cv.99P ($2n$ = 30—38) and *H. villosa* ($2n$ = 11—14). Therefore the success of somatic hybridization in wheat is much restricted by their genotypes. This may be one of the main factors limiting the progress in somatic hybridization of wheat.

In short, for the aim to regenerate the expected hybrid plants, the genetic background of both parents should be fully understood before the design of suitable combination as well as other treatments.

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