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Morphological, cellular and molecular evidences of chromosome random elimination *in vivo* upon haploid induction in maize

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ABSTRACT

The mechanism of maternal *in vivo* haploid induction is not fully understood. In this study, the young embryos were identified by morphology, cytology and simple sequence repeat (SSR) markers at different developmental stages in the cross HZ514 (sweet corn) × HZI1 (inducer). The results indicated that the low seed setting rate was determined by the inducer pollen during the process of fertilization. The mosaic endosperm kernels and the different percentages of aneuploidy, mixploidy, lagged chromosome, micronuclei, chromosomal bridge and ring chromosome were found in the cross; 7.37% of the haploid embryos carried chromosome segments from HZI1. About 1% twin seedlings resulted from the cross and were analyzed by cytology and SSR markers. Four pairs of twin seedlings had different chromosome numbers ($2n = 20$ and $2n = 10$–20) and there were some chromosome fragments from HZI1. Aneuploidy, mixploidy and the abnormal chromosomes occurred in the *in vivo* haploid induction by HZI1, which is the cytological basis for haploid induction and indicates that the inducer's chromosomes are prone to be lost during mitotic and meiotic divisions. Morphological, cellular and molecular evidences reveal that complete or partial chromosome elimination from inducer HZI1 controls the maize *in vivo* haploid induction.

DATA: The link refers to the raw data from: Morphological, cellular and molecular evidences of chromosome random elimination in vivo upon haploid induction in maize. Current Plant Biology. Raw data for phenotype, maker sequence and cytology could be directly downloaded by the link: http://dx.doi.org/10.5061/dryad.bt963

1. Introduction

The term haploid sporophyte is generally used to refer to sporophytes having the gametic chromosome number [1]. The first haploids in flowering plants were identified by Blakeslee in 1922 [2], and the doubled-haploid (DH) technology can shorten the breeding time significantly [3]. Haploids generated from a heterozygous individual and doubled to instant homozygous lines can greatly accelerate plant breeding [4–6]. For these reasons, the potential of haploids in plant breeding is recognized and considered in crop genetic improvement.

Two methods are generally used to produce haploids in plants: cells and tissues culture (*in vitro*) and genetic induction (*in vivo*). Maize haploid can also be derived through the two methods. However, tissue culture in maize is complex and greatly limited by genetic background [7,8]. Thus the method of induction-haploid *in vivo* by inducer lines, which achieves a high haploid induction frequency and is relatively simple to use, is important and widely used in maize breeding.

Several haploid-inducing lines have been developed in maize [9,10]. Stock6, with the induction rate of 0.5–3%, is one of the haploid-inducing lines discovered by Coe [11] and Sarkar and Coe [12]. However, the low induction rate could not meet the needs of breeders. When both maternal and paternal effects were detected in the process of haploid induction and the haploid-inducing character was found to be a heritable trait [9,12–15], a number of new inducers with much higher haploid-induction rate have been created by cross method among stock6, w23ig or other germplasm, such as KMS [16], WS14, ZMS [10], RWS [17], MHI [18] and HZI1 [19]. Unfortunately, the mechanism underlying *in vivo* haploid-inducing capacity in maize is not fully understood.

Researchers have focused on two possible mechanisms: parthenogenesis and chromosome elimination. Firstly, parthenogenesis was caused by the irregularities of microsporogenesis and fertilization [20–25]. All of these findings indicate that various irregularities appearing between microsporogenesis and fertilization may prevent double fertilization and stimulate division of the egg cell without fertilization. As a result of this process, a haploid

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embryo can be formed from an unfertilized egg cell. Secondly, a set of chromosome that is randomly eliminated after fertilization might be a major mechanism underlying *in vivo* haploid-induction in maize. Wedzony et al. [17] observed that 10% of the resulting embryos exhibit micronuclei of variable size after the inducer line RWS self-pollinated. Such micronuclei are characteristic of chromosome fragments being eliminated from the cell in subsequent divisions. Gernand et al. [26] found that the inducer chromosomes degenerate and fragment a few days after fertilization in interspecific crosses. Then the fragments coalesce to form the micronuclei and become eliminated from the cells within three weeks. Fischer et al. [27], Zhang et al. [19], Li et al. [28] and Zhao et al. [52] observed that a small proportion (1–3%) of haploids obtained from the cross between inducer lines and breeding materials carried several paternal chromosome segments *via* SSR markers analysis and cytogenetic makers to trace chromosomes from inducers. The results showed that some minor fragments of the inducer genome were introgressed into maternal genome of the haploids. However, Zhao et al. [52] found haploid formation with rare inducer fragment introgression. Furthermore, the aberrant fertilization mechanisms leading to haploidy may be related to mechanisms leading to hetero-fertilization [29]. Thus whether the formation of female haploid embryos results from single fertilization or from chromosome elimination remains unclear; whether haploid occur is determined by inducer or maternal materials is also unclear.

In the present study, the inducer line HZI1 derived from Stock 6 was used to induce haploids from the sweet maize pure line HZ514. The main objectives were (1) to study the characteristic of the inducer line HZI1 and identify its pollen and ear fertility; (2) to monitor the chromosome number during development of the haploid seeds after fertilization, upon induction of *in vivo* maize haploid production by HZI1. The genotype of haploid embryo was identified *via* SSR markers; and (3) to discuss the possible fundamental biological mechanisms underlying *in vivo* maternal haploid induction as well as implications of the results on improving high induction rate in maize.

2. Materials and methods

2.1. Plant materials and pollination

HZI1, a stock-6-derived haploid-induction maize line was used as the male parent, which carried the R-navajo gene that is responsible for the anthocyanin pigmentation of the endosperm and embryo. HZ514, a super sweet corn inbred line, and NA and 248, two normal corn inbred lines, were used as female parents (Table S1). The three inbred lines with colorless aleurone layer and colorless scutellum were developed by Huazhong Agricultural University (Hubei Province, China). The crosses between the inducer HZI1 and above three inbred lines were performed at Huazhong Agricultural University in 2009, and all F_1 kernels were harvested by single ear and analyzed separately. A total of 30,000 kernels were harvested from 150 ears for each cross. At the same time, HZ514 and HZI1 plants of normal development were selected for self-pollination at shedding pollens and emerging silks. The reciprocal crosses were also done between HZ514 and HZI1. HZ514 plants as receiver were crossed with NA and 248 respectively (HZ514 × NA and HZ514 × 248).

2.2. Sampling methods and cytology

2.2.1. Sample, fixation and isolation

Maize immature kernels after pollination were harvested from each ears of HZ514 × HZI1 and stored in the alcohol following a procedure similar to that used by Yang et al. [30]. From 25 to 65 h after pollination, the ears were collected every 5 h and fixed in a solution of 3:1 alcohol: glacial acetic acid for 24 h, rinsed one time every 30 min in 95% ethanol, 85% ethanol, 70% ethanol, and then stored in 70% alcohol at room temperature for further use. All the collected kernels were used for cytological observations and molecular marker analysis.

According to the method used by Herr [31] and Stelly et al. [32], the whole stain-clearing technique was used to detect the ovules development status. The ovaries were dissected in 70% ethanol, and hydrated sequentially in 50% ethanol, 30% ethanol, 15% ethanol and distilled water. After that, the ovaries were stained with diluted Enrlich's haemaloxylin dyeing liquor (primary Enrlich's haemaloxylin dyeing liquor:50% ethanol:glacial acetic acid = 1:1:1). The ovaries were rinsed 24 h with distilled water and agitated for 4–5 times in that duration. The ovaries were dehydrated one time with 15%, 30%, 50%, 70%, 85%, 95% of ethanol solutions, and then with 100% ethanol three times (dehydrated for 1 h at each step). Finally, the samples were stored in wintergreen oil for further use.

2.2.2. Microscopic examination

The cleared ovaries were put on Glass slides and observed with OLYMPUS IX71 microscope. The dissected embryos were stained with Carbol fuchsin solution for 10 min and then squashed. The samples were placed under the OLYMPUS IX71 microscope to image cell division phases and record the numbers of the chromosome present.

2.2.3. Fertility investigation

The pollen fertility of HZI1, HZ514, NA and 248 were determined as the percentage of pollen grains stained with 1% KI/I_2. The ear fertility was determined by the seed setting rate in the reciprocal cross between them.

2.3. SSR analysis

The haploid kernels and diploid kernels from the cross HZ514 × HZI1 were judged according to cytology analysis. The DNA will be extracted from the accurate haploid embryos for SSR analysis.

Genomic DNA was isolated individually from immature embryos according to a procedure similar to that used by Saghai-Maroof et al. [33]. The sequence of all SSR markers was obtained from the MaizeGDB database (www.maizegdb.org/ssr.php).

3. Results

3.1. Identification of fertility and morphology

The pollen fertility of HZI1, HZ514, NA and 248 were all normal, with over 90% regarded as fertile (Fig. S1). The pollen fertility of the haploid plants from the cross HZ514 × HZI1 were 0–38%; and the doubled plants from the haploid individual had a similar morphology and the same phenotype and genotype as the female HZ514.

In addition, 0.3% kernels with mosaic endosperm of purple aleurone and yellow shrunken without purple aleurone were found in the F_1 mature kernels from HZ514 × HZI1 (Fig. S2). The same results were also found by Zhang et al. [19].

3.2. Seed setting rate from reciprocal-cross and self-fertilization

In this study, the seed setting rate from self-fertilization or crosses among HZ514, HZI1, NA and 248 were significantly different. The seed setting rate of HZ514 self-fertilization, HZ514 × NA, HZ514 × 248 and HZI1 × HZ514 were normal. However, when HZI1 was used as the male parent for either cross or self-cross, the seed

Fig. 1. The various mating types at the developmental and mature stages. (A) The ears of HZ514 × NA, HZ514 × 248, HZ514 and HZ514 × HZI1 at 40 d after pollination; from left to right. (B) The ears of HZI1 and HZI1 × HZ514 at 40 d after pollination; from left to right. (C) The ears of HZ514 × HZI1, HZ514, HZ514 × HZI1 and HZ514 × HZI1 at 18 d after pollination; from left to right. (D) The ears of HZ514 × NA, HZ514 × HZI1, HZ514 × 248 and HZ514 at 18 d after pollination; from left to right.

setting rate was very poor and some kernels were abnormal in morphology (Fig. 1).

3.3. Fertilization status, embryo and endosperm development at nine stages after pollination in the cross HZ514 × HZI1

Three types of ovules at different sample stages (25, 30, 35, 40, 45, 50, 55, 60, 65 h) were identified in the cross HZ514 × HZI1 upon ovules whole stain-clearing: in the first type (i), the embryo and endosperm were developed (Fig. 2A); in the second type (ii), the embryo has not divided, but the endosperm was developed (Fig. 2B); and in the third type (iii), the embryo developed, but the central cell has not divided (Fig. 2C-2D). Out of 1681 ovules, the proportion of the ovules types from 25 h to 65 h after pollination was as follows: the first type was 51.45% to 57.55%, with an average of 54.19% (911); the second type was from 3.09% to 7.17%, with an average of 5.29% (89); the third type was from 37.74% to 43.57%, with an average of 40.51% (681) (Table 1);

3.4. Cytological analysis of the pollen mother cell of the HZI1, HZ514, 248 and HZ514 × HZI1

In the pollen mother cell of HZI1, only 82.3% cells had $2n = 20$ and 17.7% had $2n < 20$. In addition, 11.3% had the number of haploid cells $2n = 10$. The $2n = 10$ occupied about 10.2% of the progeny from the HZ514 × HZI1 while $2n = 20$ occupied about 86.39%. Compared with HZI1 and the cross HZ514 × HZI1, the pollen mother cell of HZ514 had 98.4% of the progeny with $2n = 20$ and only 1.6% had $2n < 20$. The pollen mother cell of 248 had 99.44% of the progeny with $2n = 20$ and only 0.56% had $2n < 20$ (Table S2).

The abnormal chromosomes were found in the pollen mother cell of the inducer HZI1, HZ514 and HZ514 × HZI1 included lagged chromosome, micronuclei and chromosome bridges. The percentages of lagged chromosomes were 1.47%, 6.83% and 8.19% in HZ514, HZI1 and HZ514 × HZI1, respectively, during meiosis; the unsynchronized chromosome condensation and division were 2.01%, 7.24% and 8.33%, respectively; Chromosome bridges and fragments occupied about 0.34%, 1.69% and 3.42%, respectively (Table 2, Fig. 3).

3.5. Cytological observation and marker analysis at 12 d after pollination in the cross HZ514 × HZI1

The frequency of cells with $2n = 10$ was 6.31%, while those with $2n = 20$ was 72.13%. More than half of the embryos of F_1 kernels had variable chromosome numbers ($2n = 9$–21), as described by Zhang et al. [19], and aneuploid embryos with $2n = 19$ and 21 were about 0.5%–1%. The kernel's endosperms with $2n = 40$, 30 or 60 were present at 1.85% and 98.15%, respectively. Most of the cells in endosperms had $2n = 30$, but there were some cells that randomly doubled to $2n = 60$ in every endosperm (Fig. 4). In addition, ring chromosomes were found in the F_1 kernels of HZ514 × HZI1 cross. The percentage of the ring chromosome was 2.13% of the total 1232 embryos (Fig. 5).

The total of 286 haploid embryos identified by cytology analysis ($2n = 10$) from HZ514 × HZI1 were analyzed by 100 SSR markers, with the diploid embryos and two parents embryos serving as the control. 92.63% haploids shared the same genomic compositions as the female HZ514 and 7.37% haploids had male chromosome fragments (Fig. 6). For example, 32 $2n = 10$ embryos were analyzed by the SSR marker umc1747 and revealed that three haploid embryos had the chromosome fragments from HZI1 (Fig. 6A). No. 21, 562, 1032 and 1632 with $2n = 10$ were found to carry male chromosome fragments identified to be bnlg1909, umc1241, bnlg1600 and umc1241, respectively (Fig. 6B–D). In addition, about 12 pair SSR markers located on chromosome 5 were detected as a heterozygous band in the $2n = 10$ embryos, which indicates a possible hot region for chromosome elimination on chromosome 5.

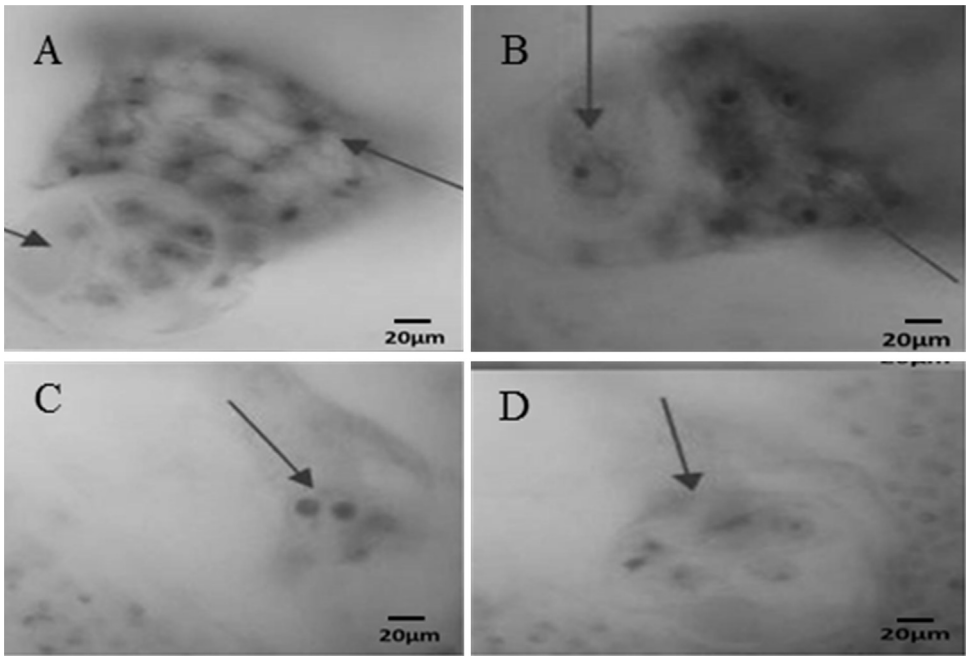

Fig. 2. Ovule whole stain-clearing at 50 h after pollination in the cross HZ514 × HZI1. (A) Arrow means dividing embryo and dividing endosperm; (B) arrow means division endosperm and no division embryo; (C) no division central cell; (D) dividing embryo.

Table 1
Three types ovules after pollination in the cross HZ514 × HZI1 at different periods.

		I	II	III	Total
25 h	Ovary no.	129	13	101	243
	%	53.09	5.35	41.56	
30 h	Ovary no.	119	16	88	223
	%	53.36	7.17	39.46	
35 h	Ovary no.	124	12	105	241
	%	51.45	4.98	43.57	
40 h	Ovary no.	85	9	70	164
	%	51.83	5.49	42.68	
45 h	Ovary no.	122	9	81	212
	%	57.55	4.25	38.21	
50 h	Ovary no.	97	10	67	174
	%	55.75	5.75	38.51	
55 h	Ovary no.	108	6	80	194
	%	55.67	3.09	41.24	
60 h	Ovary no.	68	7	49	124
	%	54.84	5.65	39.52	
65 h	Ovary no.	59	7	40	106
	%	55.66	6.6	37.74	
Total	Ovary no.	911	89	681	1681
Average	%	54.19	5.29	40.51	

Note: I, developed embryo and endosperm; II, developed embryo, undeveloped central cell; III, undeveloped egg cell, developed endosperm.

3.6. Cytology and marker analysis of twin seedlings from the progeny of the cross HZ514 × HZI1

About 1% twin seedlings were found in the progeny of the cross HZ514 × HZI1, and this percentage is higher than that in spontaneous generation [51]. There are two possible phenotypes that can be observed from the twin seedlings: (1) the two seedlings have the same phenotype and chromosomes numbers; (2) the two seedlings have different phenotypes and chromosome numbers (Fig. 7A1 and A2). Among the 20 pairs of twin seedlings, 4 pairs had different chromosome numbers ($2n = 20$ and $2n = 10–20$), and 5 twin seedlings containing $2n = 10$ and $2n = 20$. Cells with $2n = 20$ were 11 pairs (Fig. 7B1–B4). The results mean that haploid seedling and diploid seedling coexist in some twin seedlings; diploid seedlings and aneuploidy or mixoploid coexist in some twin seedlings; the rest twin seedlings are diploid.

Table 2
Frequency of the abnormal chromosomes in HZ514, HZI1 and HZ514 × HZI1.

Material and cell numbers	Total (%)	Lagged chromosome (%)	Unsynchronized chromosome condensation and division (%)	Chromosome bridge (%)
HZ514 (2682)	3.48	1.47	2.01	0.34
HZI1 (2265)	14.06	6.83	7.24	1.69
HZ514 × HZI1 (2570)	16.52	8.19	8.33	3.42

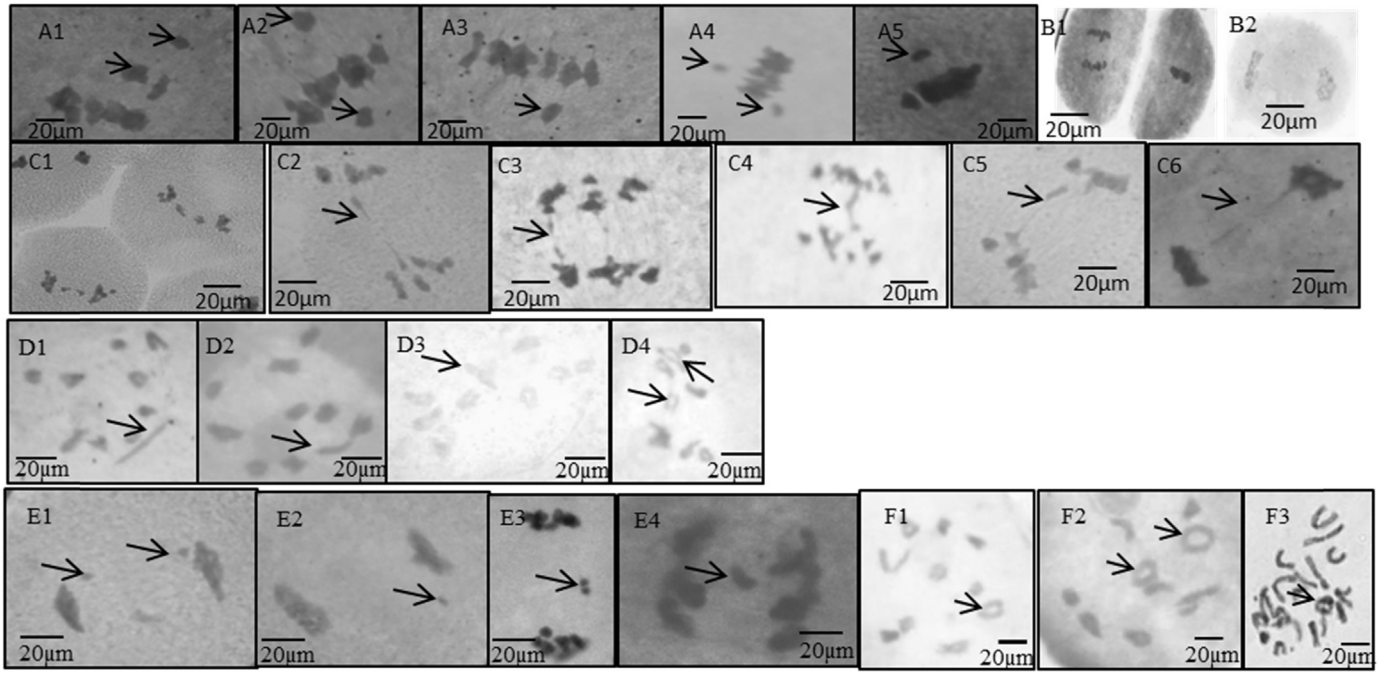

Fig. 3. Various chromosomes morphology after pollination in the cross HZ514 × HZI1. (A1–A5) Lagged chromosome; (B1–B2) unsynchronized division; (C1–C6) chromosome bridge in late mitosis; (D1–D4) unsynchronous chromosome; (E1–E4) micronuclei at telophase; (F1–F3) cycle chromosome.

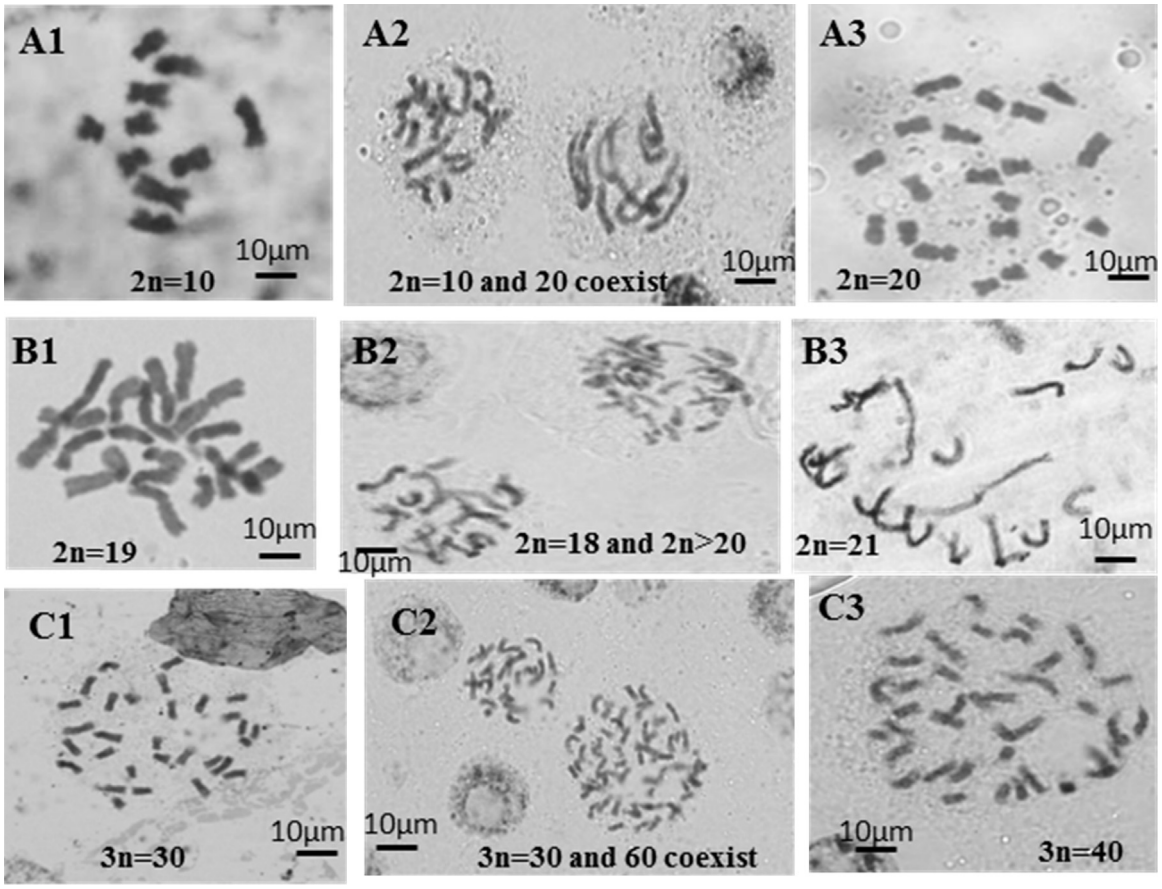

Fig. 4. Cytology of F_1 kernels from HZ514 × HZI1 cross at 12 days after pollination. (A1–A3) the embryo of the F_1 kernels with $2n = 10$ (A1), 10 and 20 coexisting (A2), 20 (A3); *Bar* 10 μm. (B1–B3) Aneuploid embryo of the F_1 kernels with $2n = 19$(B1), $2n = 18$ and $2n > 20$ coexisting (B2), $2n = 21$(B3); *Bar* 10 μm. (C1–C3) the endosperm of the F_1 kernels, with $3n = 30$ (B1), 30 and 60 coexist (B2), 40 (B3); *Bar* 10 μm.

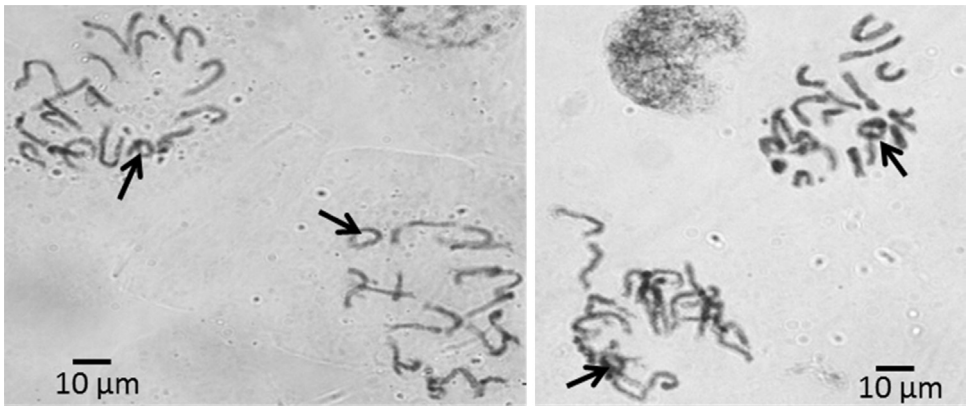

Fig. 5. Cycle chromosome of F_1 kernels from HZ514 × HZI1 cross at 12 days after pollination.

The DNA of the 20 twin seedlings were extracted and analyzed by 100 SSR markers located on ten maize chromosomes. Some chromosome fragments were from HZI1 in $2n = 10$ seedlings and $2n = 11–19$ seedlings, whereas no chromosome fragment was from HZI1 in $2n = 20$ seedlings. For example, five twin seedlings were analyzed by umc1447 and umc2030 markers and heterozygous bands were found in three seedlings (Fig. 7C1 and C2)

4. Discussion

Measurement of the seed setting rate from reciprocal-cross and self-fertilization among HZI1, HZ514, NA and 248 at different development stages revealed that the low seed setting rate is only detected when the inducer line is male. About 36%–50% kernels from HZ514 × HZI1 were abortive (Fig. 1), indicating that the phenomenon may be caused by inducer pollen. However, the pollen fertility of the inducer and other materials were normal and over 90% were fertile (Fig. S1). Lin et al. [34] speculated that abortive grain was related with the excrescent polar nuclei in megaspore. According to the results in this study, the main reason may be that the process of fertilization was abnormal, in that the two sperms of inducer were not normal; and the low setting rate was not caused by the sperm number, but may be caused instead by alterations in the chromosomal structure or cell organelles in sperms during the second mitosis in microspores. The haploid induction should also be determined during this abnormal process.

$2n = 10$ kernels occupied about 10.2% in the cross HZ514 × HZI1, with the final haploid induction frequency at 6.31%, lower than that in the development after pollination. This result indicates that some kernels which are aneuploid and mixploid were sterile during the development process and the chromosomes from HZI1 may be completely lost during the development process of embryos. Thus we should take into account that the seed setting rate of the inducer is lower whether self-crossed or used as male [35]. This information will be helpful to breeders during the process of higher frequency inducer selection or haploid breeding [36].

The F_1 kernels of the cross HZ514 × HZI1 should be flint corn. However, over 0.3% of the mosaic endosperm kernels were found in the progenies of the cross, and these kernels exhibited different proportions of the sweet shrunken endosperm in some ears (Fig. S2). The same results were also found by Zhang et al. [19], and Zhao et al. [52] and Xu et al. [53]. These observations could

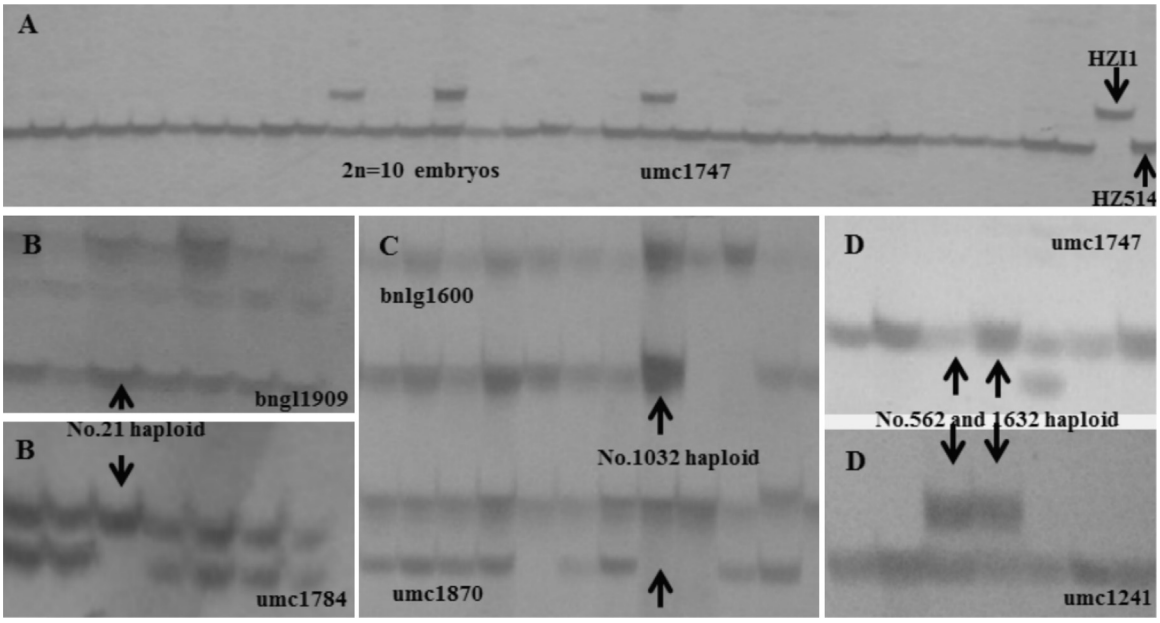

Fig. 6. SSR analysis of haploid embryos in the cross HZ514 × HZI1 at 12 days after pollination. (A) 32 $2n = 10$ embryos were analyzed by the SSR marker umc1747 and three haploid embryos with inducer's chromosome fragments; (B) arrow is No. 21 with haploid embryo, was analyzed by umc1784 and bnlg1909, the inducer's chromosome fragments was found in No. 21 by bnlg1909; (C) arrow is No. 1032 with haploid embryo, was analyzed by umc1870 and bnlg1600, the inducer's chromosome fragments was found in No. 1032 by bnlg1600; (D) arrows are Nos. 562 and 1632 with haploid embryos, which carried male fragments identified by marker umc1241 and no male fragments by marker umc1747.

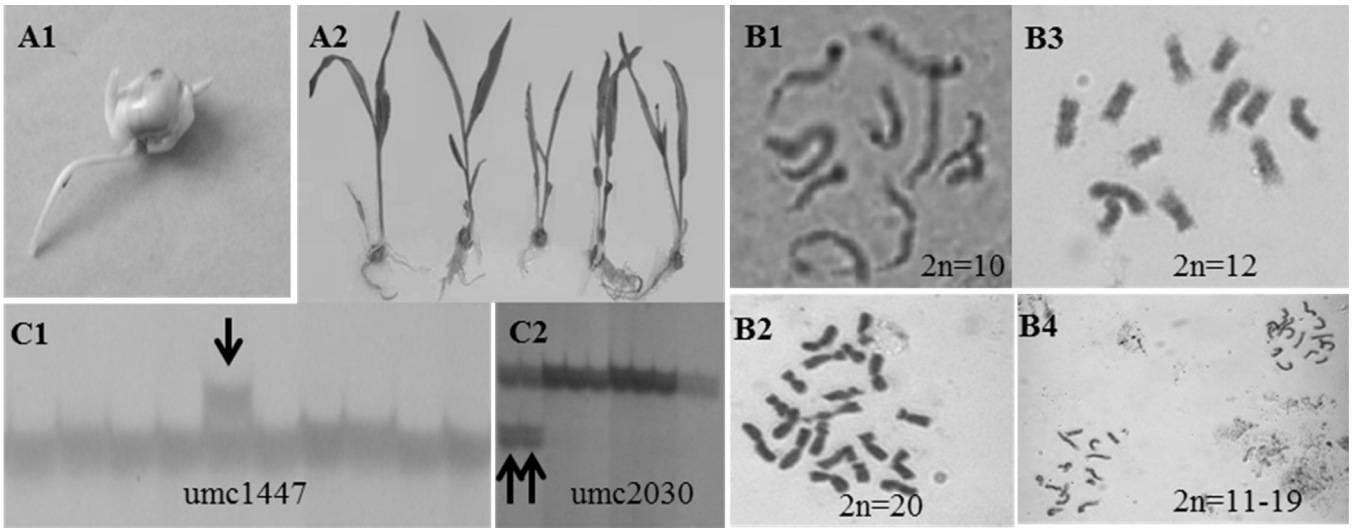

Fig. 7. The genotype and phenotype of twin seedlings in the HZ514 × HZI1 cross. (A1 and A2) twin seedlings; (B1) the small seedlings with $2n = 10$; (B2) the large seedlings with $2n = 20$; (B3 and B4) the seedlings are aneuploidy or mixoploidy with $2n = 12$ and $2n = 11–19$; (C1 and C2) The seedlings with $2n = 10$ carried male fragments identified by marker umc1447 and umc2030.

be accounted for by the double recessive sweet endosperm and yellow aleurone layer organization. The mosaic mixoploid traits were caused by the complete elimination of the paternal chromosome in one daughter cell from the first mitosis of the primary endosperm nuclei during the process of cell division. In addition, the whole ears phenotypes of the induced progeny showed that the different ratios of mosaic kernels were caused by the different elimination times of paternal chromosomes during the endosperm developmental process after cross fertilization.

We observed three types of ovules with different embryos and endosperms after pollination at different development periods in the cross HZ514 × HZI1 (nine stages, from 25 to 65 h). Kernels obtained from the cross-pollinated ears also were classified into three categories: (i) normal kernels with normal embryos and endosperms, normal diploids and haploids were included; (ii) endosperm abortion, with shrunken endosperms or defective kernels; (iii) embryo abortion, with normal endosperms but without embryos. Based on the fertilization status of the embryos and endosperms in the cross, we speculate that the process of fertilization and the different developmental stages must be abnormal and that this was the main reason for the haploidy (Table 1 and 2; Fig. 2). Similar results were found by Bylich et al. [21] and Coe et al. [37], but they did not provide possible mechanisms; Rotarenco and Eder [25] detected a much higher rate of heterofertilization when the haploid inducer MHI was used instead of the normal line. Xu et al. [53] reported that the different kernel phenotypes are most probably caused by the *sed1* locus, they speculated that the defective kernels and haploids are caused by the same genetic mechanism. However, we could not speculate what caused the change in the number of sperm in synergid in this study. Is it because the sperm stayed in synergid and did not make it to the egg cell, or that fertilization was finished and could not proceed to egg cell division? Did the egg cell stay somewhere at the megaspore stage? We could not confirm any of these possibilities by whole stain-cleaning method and optical microscope alone. Whether parthenogenesis occurred or not need further study, but our results support that the abnormal process of fertilization must be one reason for the maternal haploid induction.

The paternal haploid could be found in the progeny of the inducers [38]. The percentage of inducer pollen cells with aneuploidy was significantly higher than other normal inbred lines. Similar results were also identified for microsporocytes of the inducer MHI [22] and radicle cells of the inducer [19]. Although the pollen fertility of the inducer was normal, the chromosome ploidy variation was the main reason that caused high frequency of heterofertilization. Lagged chromosomes, the micronuclei and chromosome bridges were often used as direct evidences of chromosome elimination and haploid production in inter-, intra-specific hybridizations in crops [17,39,40] also found micronuclei in the maize haploid inducer RWS. In this study, the abnormal chromosomes, such as lagged chromosome, micronuclei and chromosome bridges, indicate that chromosome elimination occurred in the primordium of the inducer after self-pollination and that chromosome segregations were not synchronous and equal during cell meiosis. According to the results, chromosome random elimination should occur during haploid induction. The aneuploidy should be induced by partial elimination of chromosomes from HZI1. About 1.85% endosperms had $2n = 40$ which may be caused by chromosome randomly partial elimination. Remarkably, about 2.13% ring chromosome was observed in 1232 embryos (Fig. 5). The ring chromosome is formed after chromosome deletion or elimination. Thus our results indicate that random elimination of chromosome occurs during the *in vivo* haploid induction by inducers in maize.

Khokhlov et al. [41] pointed out that diploid cells exist in any tissues of haploid and Wei et al. [42] observed that 80% plants had doubled haploid cells. There were some cells that always randomly doubled to $2n = 60$ in endosperms (Fig. 4 C2), which was similarly observed with haploid embryos. However, the doubling frequency of haploid embryos is lower than in endosperm. In this study, 80% of the identified megaspores had fertilized polar nucleus and normally divided endosperm. These also contained multi nucleoli which may have triggered the random doubling of the endosperm.

Our results from the genome-wide SSR markers demonstrated that the introgression of genetic element from the inducer HZI1 was occurred during the process of chromosome elimination. The results of cytological analysis and genotype analysis based on twin seedlings also indicate that chromosome introgression or elimination occurs during the haploid induction by the inducer. Similar results were found by Fischer et al. [27], Zhang et al. [19] and Li et al. [28]; but Zhao et al. [53] found haploid formation with rare inducer fragment introgression, the discrepancy might be due to different genetic background, different markers and marker numbers. One major QTLs controlled haploid induction rate were identified on chromosome 5 [43], and haploid embryos with male

chromosome fragment could be identified by 12 pairs SSR markers on chromosome 5. This indicates the presence of one hot region of chromosome introgression or exchange which may control the haploid induction rate.

The chromosome elimination mechanism is similar to that in genome wide hybridizations and probably controlled by some genetic factors in the inducers [39,40,43–50,52,53]. Aneuploidy, mixploidy and the abnormal chromosomes observed upon *in vivo* haploid induction by inducer were found in the pollen cells, microsporocytes and radicle cells of inducers. This is the cytological basis for haploid induction, suggesting that the inducer's chromosomes were prone to be lost during mitotic and meiotic divisions after the inducers were crossed with other lines. The morphological, cellular and molecular evidences suggest that maize *in vivo* haploid induction is controlled by complete or partial chromosome elimination from inducer HZI1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cpb.2014.04.001.

References

[1] C.E. Palmer, W.A. Keller, Overview of haploidy Haploids in Crop, Improvement II, vol. 56, Springer, Heidelberg, Germany, 2005, pp. 3–9.
[2] A.F. Blakeslee, J. Belling, M.E. Farnham, A.D. Bergner, A haploid mutant in *Datura stramonium*, Science 55 (1922) 646–647.
[3] S. Guha, S. Maheshwari, In vitro production of embryos from anthers of *Datura*, Nature 204 (1964) 497.
[4] B.P. Forster, W.T.B. Thomas, Doubled haploids in genetics and plant breeding, Plant Breed Rev. 25 (2005) 57–88.
[5] B.P. Forster, E. Heberle-Bors, K.J. Kasha, A. Touraev, The resurgence of haploids in higher plants, Trends Plant Sci. 12 (8) (2007) 368–375.
[6] M. Ravi, S.W. Chan, Haploid plants produced by centromere-mediated genome elimination, Nature 464 (2010), http://dx.doi.org/10.1038/nature08842.
[7] M. Beckert, in: Y.P.S. Bajaj (Ed.), Advantages and disadvantages of the use of in vitro/in situ produced DH maize plants, Biotechnol. Agric. For. 25 (1994) 201–213.
[8] O.A. Shatskaya, E.R. Zabirova, V.S. Shcherbak, M.V. Chumak, Mass induction of maternal haploids in corn, Maize Genet. Coop. Newsl. 68 (1994) 51.
[9] K.R. Sarkar, S. Panke, J.K.S. Sachan, Development of maternal-haploidy-inducer lines in maize (*Zea mays* L.), Indian J. Anim. Sci. 4 (1972) 781.
[10] S.T. Chalyk, Properties of maternal haploid maize plants and potential application to maize breeding, Euphytica 79 (1994) 13–18.
[11] E.H. Coe, A line of maize with high haploid frequency, Am. Nat. 93 (1959) 381–382.
[12] K.R. Sarkar, E.H. Coe, A genetic analysis of the origin of maternal haploids in maize, Genetics 54 (1966) 453–464.
[13] P. Lashermes, M. Beckert, Genetic control of maternal haploidy in maize (*Zea mays* L.) and selection of haploid inducing lines, Theor. Appl. Genet. 76 (1988) 405–410.
[14] M.A. Aman, K.R. Sarkar, Selection for haploidy inducing potential in maize, Indian J. Genet. 38 (1978) 452–457.
[15] E.H. Coe, K.R. Sarkar, The detection of haploids in maize, J. Hered. 55 (1964) 231–233.
[16] V.S. Tyrnov, A.N. Zavalishina, Inducing high frequency of matroclinal haploids in maize, Dokl. Akad. Nauk SSSR 276 (1984) 735–738.
[17] M. Wedzony, F.K. Rober, H.H. Geiger, Chromosome elimination observed in selfed progenies of maize inducer line RWS, in: XVIIth International Congress on Sex Plant Rep, Maria Curie-Sklodowska University Press, Lublin, 2002, p. 173.
[18] J. Eder, S. Chalyk, In vivo haploid induction in maize, Theor. Appl. Genet. 104 (2002) 703–708.
[19] Z.L. Zhang, F.Z. Qiu, Y.Z. Liu, K.J. Ma, Z.Y. Li, S.Z. Xu, Chromosome elimination and in vivo haploid induction by stock 6-derived inducer line in maize (*Zea mays* L.), Plant Cell Rep. 27 (2008) 1851–1860.
[20] S. Hu, Male germ unit and sperm heteromorphism: the current status, Acta Bot. Sin. 32 (1990) 230–240.
[21] V.G. Bylich, S.T. Chalyk, Existence of pollen grains with a pair of morphologically different sperm nuclei as a possible cause of the haploid-inducing capacity in ZMS line, Maize Genet. Coop. Newsl. 70 (1996) 33.
[22] S.T. Chalyk, A. Baumann, G. Daniel, J. Eder, Aneuploidy as a possible cause of haploid-induction in maize, Maize Genet. Coop. Newsl. 77 (2003) 29.
[23] M.T. Chang, Preferential fertilization induced from Stock 6, Maize Genet. Coop. Newsl. 66 (1992) 99–100.
[24] N.K. Enaleeva, V.S. Tyrnov, L.P. Selivanova, A.N. Zavalishina, Single fertilization and the problem of haploidy induction in maize, Dokl. Biol. Sci. 353 (1996) 225–226.
[25] V.A. Rotarenco, J. Eder, Possible effect of heterofertilization on the induction of maternal haploids in maize, Maize Genet. Coop. Newsl. 77 (2003) 30.
[26] D. Gernand, T. Rutten, A. Varshney, C. Bruss, R. Pickering, F. Matzk, A. Houben, Selective chromosome elimination in embryos of interspecific crosses, in: 15th International Chromosome Conference (poster), September 5–10, Brunel University, London, 2004.
[27] E. Fischer, Molekulargenetische untersuchungen zum vorkommen paternaler DNA-übertragung bei der in-vivo-haploideninduktion beimais (*Zea mays* L.) (PhD dissertation), University of Hohenheim, Grauer Verlag, Stuttgart, 2004.
[28] L. Li, X. Xu, W. Jin, S. Chen, Morphological and molecular evidences for DNA introgression in haploid induction via a high oil inducer CAUHOI in maize, Planta 230 (2009) 367–376.
[29] A. Kato, Induced single fertilization in maize, Sex. Plant Reprod. 10 (1997) 96–100.
[30] X. Yang, X. Dai, Observation of mitosis and meiosis in rice cells by simple squash method, Agric. Sci. Technol. 10 (5) (2009) 96–99.
[31] J.M. Herr, A new clearing-squash technique for the study of ovule in angiosperms, Am. J. Bot. 58 (1971) 785–790.
[32] D.M. Stelly, S. Peloquin, R.G. Palmer, Mayer's hemalummthyl salicylate: a stain-clearing technique for observations within whole ovules, Stain Technol. 59 (1984) 155–161.
[33] M.A. Saghai-Maroof, K.M. Soliman, R.A. Jorgensen, R.W. Allard, Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics, Proc. Natl. Acad. Sci. 81 (1984) 8014–8018.
[34] B.Y. Lin, Ploidy barrier to endosperm development in maize, Genetics 107 (1984) 103–115.
[35] J.L. Kermicle, Pleiotropic effects on seed development of the indeterminate gametophyte gene in maize, Am. J. Bot. 58 (1971) 1–7.
[36] V.A. Rotarenco, G. Dicu, D. State, S. Fuia, New inducers of maternal haploids in maize, Maize Genet. Coop. Newsl. 84 (2010), http://www.agron.missouri.edu/mnl/84/PDF/15rotarenco.pdf
[37] E.H. Coe, M.G. Neuffer, Darker orange endosperm color associated with haploid embryos: Y1 dosage and the mechanism of haploid induction, Maize Genet. Coop. Newsl. 79 (2005) 7.
[38] J.L. Kermicle, Androgenesis conditioned by a mutation in maize, Science 166 (1969) 1422.
[39] K.J. Kasha, K.N. Kao, High frequency haploid production in barley (*Hordeum vulgare* L.), Nature 225 (1970) 874–876.
[40] D.A. Laurie, M.D. Bennett, The timing of chromosome elimination in hexaploid wheat and maize crosses, Genome 32 (1989) 953–961.
[41] S.S. Khokhlov, V.S. Tyrnov, E.V. Grishina, N.I. Davoyan, Haploidy and Breeding, Nauka, Moscow, 1976, pp. 221 (in Russian).
[42] J.J. Wei, H.M. Li, Z.Z. Liu, Cytology study of maize Haploid, J. Baoding Teach. Coll. 16 (4) (2003) 21–23 (in Chinese).
[43] P. Vanessa, S. Wolfgang, M. George, A.N. Gary, E.M. Albrecht, Development of in vivo haploid inducers for tropical maize breeding programs, Euphytica 185 (2012) 481–490.
[44] D.A. Laurie, The frequency of fertilization in wheat × pearl millet crosses, Genome 32 (1989) 1063–1067.
[45] P. Barret, M. Brinkmann, M. Beckert, A major locus expressed in the male gametophyte with incomplete penetrance is responsible for in situ gynogenesis in maize, Theor. Appl. Genet. 117 (2008) 581–594.
[46] S. Deimling, F.K. Rober, H.H. Geiger, Methodik und genetic der in-vivo- haploideninduktion bei mais, Vortr. PXanzenzuchtg. 38 (1997) 203–224.
[47] D. Gernand, T. Rutten, A. Varshney, M. Rubtsova, S. Prodanovic, C. Bruß, J. Kumlehn, F. Matzk, A. Houben, Uniparental chromosome elimination at mitosis and interphase in wheat and pearl millet crosses involves micronucleus formation, progressive heterochromatinization, and DNA fragmentation, Plant Cell 17 (2005) 2431–2438.
[48] D. Gernand, T. Rutten, R. Pickering, A. Houben, Elimination of chromosomes in *Hordeum vulgare* 9H, bulbosum crosses at mitosis and interphase involves micronucleus formation and progressive heterochromatinization, Cytogenet. Genome Res. 114 (2006) 169–174.
[49] K. Mochida, H. Tsujimoto, T. Sasakuma, Confocal analysis of chromosome behavior in wheat × maize zygotes, Genome 47 (2004) 199–205.
[50] O. Riera-Lizarazu, H.W. Rines, R.L. Phillips, Cytological and molecular characterization of oat × maize partial hybrids, Theor. Appl. Genet. 93 (1996) 123–135.
[51] L. Li, X. Dong, X. Xu, L. Liu, C. Liu, S. Chen, Observation of twin seedling in maternal haploid induction in maize, J. China Agric. Univ. 17 (5) (2012) 1–6.
[52] X. Zhao, X. Xu, H. Xie, S. Chen, W. Jin, Fertilization and uniparental chromosome elimination during crosses with maize haploid inducers, Plant Physiol. 163 (2013) 72–731.
[53] X. Xu, L. Li, X. Dong, W. Jin, A.E. Melchinger, S. Chen, Gametophytic and zygotic selection leads to segregation distortion through in vivo induction of a maternal haploid in maize, J. Exp. Bot. 64 (4) (2013) 1083–1096.