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Chromosome elimination and in vivo haploid production induced by Stock 6-derived inducer line in maize (*Zea mays* L.)

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Abstract In vivo haploid production induced by inducer lines derived from Stock 6 is widely used in breeding program of maize (*Zea mays* L.), but the mechanisms behind have not yet been fully understood. In this study, average frequency of haploid induction in four inbred lines by Stock 6-derived inducer line HZI1 was above 10%. About 0.2% kernels from the cross Hua24 × HZI1 had mosaic endosperm showing yellow shrunken parts from Hua24 to normal parts with purple aleurone from HZI1. Individual lagged chromosomes and micronuclei were observed in mitotic cells of ovules pollinated by HZI1. Above 56.4% of the radicles from the kernels with purple aleurone and colorless embryos were mixoploid ($2n = 9$–21), and more than 45.22% cells were haploid cells ($2n = 10$) in three crosses. More than 62.5% of the radicles from the kernels with purple aleurone and purple embryos were mixoploid ($2n = 9$–21) having 54.27% cells with $2n = 20$. SSR analysis showed that all haploids from the cross Hua24 × HZI1 shared the same genomic compositions as Hua24 except for plants Nos. 862 and 857 with some polymorphic DNA bands. The results revealed that chromosome elimination after fertilization caused the haploid production in maize.

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Introduction

It takes 7–8 generations to obtain a maize inbred line and the plants were still not 100% pure in traditional breeding. Doubled-haploid (DH) technology improves breeding efficiency by generating inbred lines with 100% purity and genetic uniformity in just two generations. DH lines make it easy to carry genetic studies and shorten the breeding time significantly. Maize (*Zea mays* L.) is a typical diploid plant ($2n = 20$), but haploid individuals ($2n = 10$) occur naturally at a rate of one per 1,000 kernels (Chase 1949). Maize haploid can be derived either through tissue culture technique (in vitro) or through genetic induction (in vivo). Anther culture in maize is a highly complex and expensive method with low plantlet regeneration rate, which is dependent on genetic background (Beckert 1994; Shatskaya et al. 1994) and is greatly limited for the application in breeding programs. Nowadays, in vivo haploid induction by inducer lines is widely used by most breeders for its high frequency, simple operation and inexpensiveness.

There are two modes of in vivo haploid induction in maize, leading to maternal and paternal haploids, respectively. The genomes of maternal haploids originate from the maternal parent and those of paternal haploids come from the paternal parent. Coe (1959) discovered a haploid inducer, Stock 6, and 1–2% of the progeny was maternal haploids when it was used as pollinator. Kermicle (1969) obtained 0–2% paternal haploids when the W23 indeterminate gametophyte (ig) line was used as female parent. However, the rate of haploids obtained with Stock 6 and

W23 ig was still low and depended on the genotypes (Lashermes and Beckert 1988). The haploid-inducing capacity of the inducer can be increased by selection (Sarkar et al. 1972). To date, a number of new inducers with higher haploid-induction rate have been created (Eder and Chalyk 2002), such as WS14 (Lashermes et al. 1988), ZMS (Chalyk 1994), KMS (Tyrnov and Zavalishina 1984), MHI (Eder and Chalyk 2002), and RWS (Röber et al. 2005).

The mechanism of in vivo haploid induction has not yet been full understood until now (Eder and Chalyk 2002; Röber et al. 2005). Several hypotheses have been put forward to explain the haploid formation through in vivo haploid induction in maize. Firstly, the irregularities during microsporogenesis and fertilization may be involved in haploid induction. Hu (1990) found the difference between the transmission velocities of two sperms in one microspore, and the sperm with high velocity fertilized normally, while the one with low velocity missed the fertilization, which broke the normal double fertilization and developed the kernels with a haploid embryo (Enaleeva et al. 1996). Chang (1992) suggested two irregular cases occurring in the maize double fertilization. Primarily, the polar nucleus is fertilized, while the egg cell remains unfertilized and develops into embryos with the cell division of the fertilized polar nucleus. Alternatively, the egg cell is destroyed when the pollen tube is entering into embryo sacs, one of the sperms fertilizes the polar nucleus and other develops into haploid embryos. Bylich and Chalyk (1996) detected pollen grains with a pair of morphologically different sperm nuclei in ZMS inducer line, as a result, one of the sperms can fertilize normally, but another does not. Rotarenco and Eder (2003) detected a more than three time higher rate of heterofertilization when haploid inducer was used as pollinator compared to a normal inbred line. Chalyk et al. (2003) observed above 15% aneuploidy microsporocyte in the inducer MHI and only about 1% in two inbred lines used as checks. Briefly, the abnormality in microsporogenesis and fertilization may be the reasons of haploid induction. Coe and Neuffer (2005) also supported this hypothesis. Secondly, chromosome elimination after fertilization might be the major mechanism in maize in vivo haploid induction. Wedzony et al. (2002) studied ovaries of inducer line RWS during the first 20 days after self-pollination. In about 10% of the embryos, micronuclei of variable size were found in the cytoplasm of every cell of the shoot primordium. Such micronuclei generally were the symbol of chromosome elimination from the cell in subsequent divisions (Kasha and Kao 1970). Fischer (2004) showed that a small proportion (1–2%) of haploids obtained from the cross between the inducer line and a broad-based sample of breeding materials carried one, seldom several paternal chromosome segments through SSR markers analysis. It indicated that a small fragment from the inducer genome can be transferred into maternal genome of the haploids (Röber et al. 2005).

In present study, we find that chromosome elimination after fertilization leads to in vivo maize haploid production induced by inducer line HZI1 derived from Stock 6. Cells with variable chromosome numbers appear in the radicles of the F_1 kernels from crosses with inducer HZI1, and individual lagged chromosomes and micronuclei are observed in the pollinated ovules by HZI1. The F_1 kernels with mosaic endosperm consisting of normal part with purple aleurone and sweet shrunken part without purple aleurone are also observed.

Materials and methods

Plant materials

An inducer line HZI1 derived from Stock 6 with *R-nj* (*R-nj*: R-navajo, a domiant gene on chromosome 10 for purple aleurone and purple embryo) and *Sh2* (the dominant gene of *sh2* on chromosome 3 for normal endosperm) was used as male parent, and it has normal endosperm with purple aleurone and purple embryo. Three normal inbred lines were used as female parents: HZ124b, HZ85, HZ141 with *r(r*, recessive gene of *R-nj)* and *Sh2*, and with the normal endosperm with colorless aleurone and colorless embryo, as well as a sweet corn inbred line Hua24 with *r (r*, recessive gene of *R-nj)* and *sh2(sh2*, shrunken2, showing shrunken endosperm), and with shrunken endosperm with colorless aleurone and colorless embryo.

The crosses between the inducer HZI1 and above inbred lines were made in field, respectively, and all F_1 kernels were harvested by single ear and analyzed separately. We identified haploid kernels by using the system of dominant anthocyanin marker genes (Chase 1969; Nanda and Chase 1966). In this system, the expression of the R-nj gene provided an anthocyanin pigmentation of the embryo and the endosperm. Kernels with purple aleurone and colorless embryo were putative haploids and the kernels with purple aleurone and purple embryo were hybrid kernels. The kernels with colorless aleurone and purple embryo were defect kernels and the kernels with colorless aleurone and colorless embryo were from pollen contamination.

Cytology

Twenty radicles of each parents and 50 radicles of each phenotype of F1 kernels from the crosses of HZI1 as male parent with Hua24, HZ124b, HZ141, and HZ85, were collected after germination for three days. In addition, 17

radicles of each phenotype of F1 kernels of the cross HZ85 × HZI1 collected at 3, 5, 7 days after germination respectively. All the collected radicles were used to determine chromosome numbers.

The radicles preparations followed the methods reported by Kindiger and Beckett (1983) except that the collected radicles were pretreated with 2 mM 8-hydroxyquinoline for 5 h at 22°C. Chromosome numbers were determined following the procedure by Li et al. (1995). One hundred ovaries of Hua24 36 h after pollinated by HZI1 were collected and fixed in a solution of 3:1 ethanol:acetic acid for 24 h then transferred to fresh solution and stored at −20°C for cytological observations.

Morphology and fertility investigation

The inducer and inbred lines were planted in the field at Wuhan in spring and Hainan Island in winter in 2006. The seedlings after radicles collection were transplanted in the field in spring of 2007 for molecular and morphological investigation. The putative haploid plants from morphological observation obtained from the cross Hua24 × HZI1 were transplanted adjacent to the female plants Hua24 to facilitate the traits comparison in field, and several traits were measured, such as plant height, ear height, ear leaf length, ear leaf width, tassel length and branches etc., as described by Augers et al. (2004). The data were analyzed by *t* test (Excel 2003) and clustered using SAS V8.02 (SAS Institute Inc. Cary, NC, 1991–2001).

The pollen fertility was determined as the percentage of pollen grains stained with 1% KI/I_2. More than 400 pollen grains were counted for each plant. Female inflorescence fertility of haploid plants was determined by the seed setting after pollinated by inbred line Hua24.

SSR analysis

Maize genomic DNA was isolated from expanding leaves of plants according to CTAB procedure (Saghai-Maroof et al. 1984). SSR analysis was made in the haploid plants of the crosses Hua24 × HZI1 and HZ124b × HZI1 and both their parents, by the protocol of Qiu et al. (2007), and clustering was made using NTsys Version2.0. Sequences of all SSR markers were obtained from the MaizeGDB database (http://www. maizegdb.org/ssr.php).

Results

Frequency, morphology and fertility of haploids

The frequency of haploid induction by HZI1 was averagely above 10% identified by the *R-nj* genetic markers in kernels, which ranged from 8.19 to 13.89% for different female parents (Table 1). Remarkably, about 0.2% kernels in the F_1 kernels from the cross Hua24 × HZI1 had mosaic endosperm of purple aleurone half and yellow shrunken half without purple aleurone (Fig. 1c), for the kernels of Hua24 had shrunken endosperm with colorless aleurone (Fig. 1a), while the kernels of inducer HZI1 had normal endosperm with purple aleurone (Fig. 1b). The mosaic kernels had different sizes of sweet shrunken endosperm in the top centre of the kernels (Fig. 1d1, d2, d3).

Table 1 Haploid frequencies identified by the expression of *R-nj* gene in four crosses

Crosses	Total kernels	Average haploid (%)
Hua24 × HZI1	880	12.65
HZ124b × HZI1	2,182	11.91
HZ141 × HZI1	1,976	8.19
HZ85 × HZI1	909	13.89

Compared with the diploid plants, haploid seedlings of Hua24 were short in stature and grew slowly with distinctive narrow leaves, slim and weak stems and lightly yellow leaves (Chase 1964) (Fig. 1e1, e2). After flowering, there were significantly differences between haploid and diploid plants in plant height, ear height, ear leaf length, ear leaf width, tassel length (Table 2; Fig. 1). The florets of the haploid tassels did not open or rarely contained pollen grains after opening (Fig. 1f3).

The morphological clustering analysis of 21 haploid plants and 22 Hua24 plants showed that all 43 plants were mainly classified into two types, haploid and diploid (data not shown). Haploid No. 857 was clustered into a new type, which might correspond to its morphology. Its tassel was different from other haploid plants by showing the red floret and some pink anthers and less tassel branches (Fig. 1f4). The length of its ear leaf was 37 cm, much shorter than the average (56 cm) of other haploid plants ($t = 32.76$, $Pr > |t|$, <0.0001), the width was 3.7 cm, narrower than the average (6.5 cm) of others ($t = 19.52$, $Pr > |t|$, <0.0001), and the tassel branches was 5, significantly fewer than the average 20.7 of others ($t = 16.40$, $Pr > |t|$, <0.0001).

The pollen fertility of the haploid plants from the cross Hua24 × HZI was 0–40% (Fig. 2b1, b2) except for 41% of No. 858 and 42% of No. 867. No seeds were produced by all haploids after pollinated by Hua24, except for 13 seeds from plant No. 857 and 3 seeds from plant No. 858.

Twenty-five haploid plants from the cross HZ124b × HZI1 were morphologically very similar and had the same phenotype as the female HZ124b.

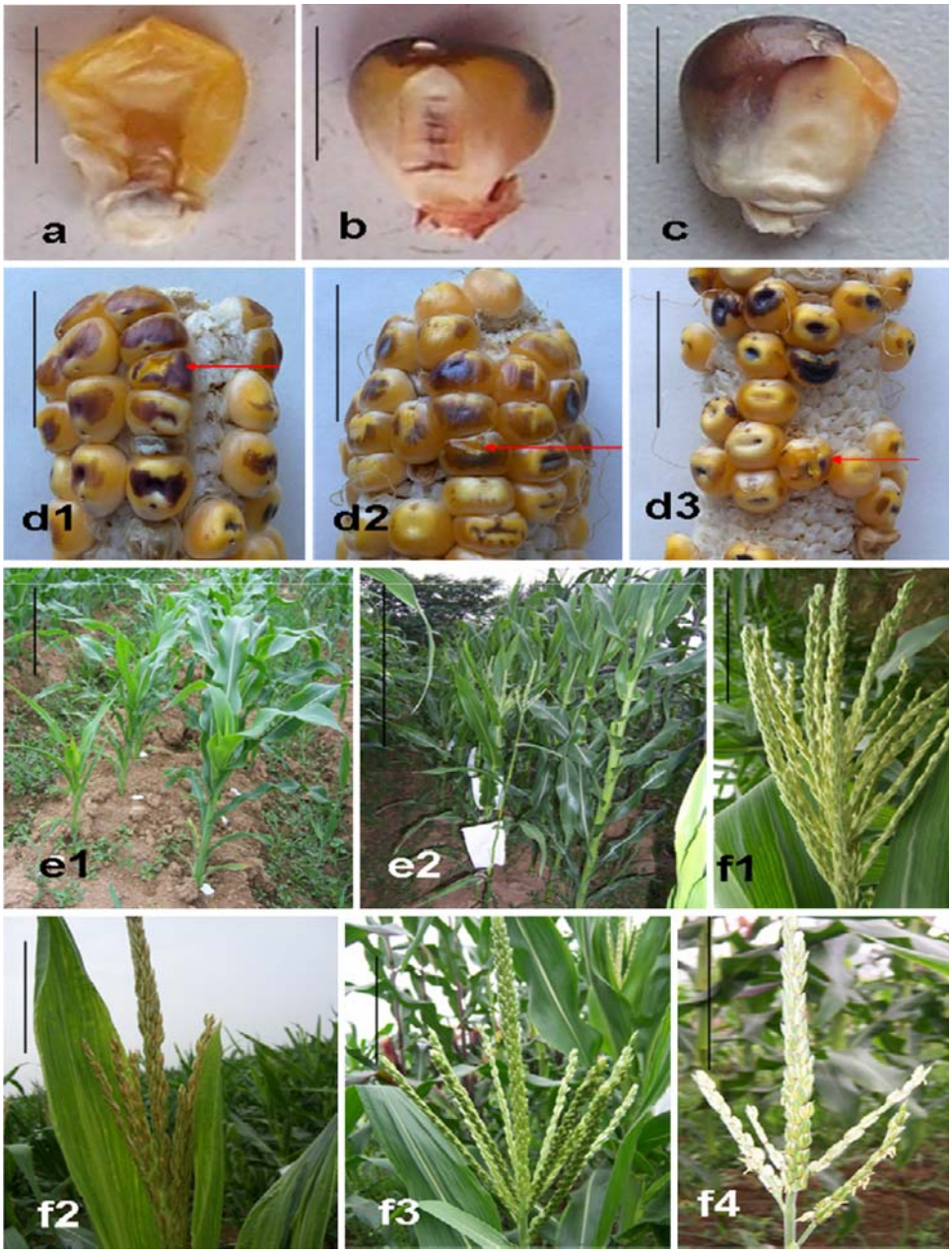

Fig. 1 Morphology of haploids from the cross Hua24 × HZI1. **a**, **b** The kernels of the sweet corn inbred line (**a**), HZI1 (**b**). **c** The mosaic endosperm kernel with half normal endosperm and half with sweet shrunken endosperm. *Bar* 0.5 cm. **d1**, **d2**, **d3** The mosaic endosperm kernels with different portions of the sweet shrunken endosperm in the ear (*arrows*). *Bar* 0.5 cm. **e1**, **e2** Morphology of the haploid plants from Hua24 × HZI1 cross and Hua24 in the field. **e1** Young haploid (*left*), diploid (*right*) plants. *Bar* 10 cm. **e2** Adult haploid (*left*) and diploid (*right*) plants. *Bar* 90 cm. **f1**–**f4** Tassels of Hua24 (**f1**), HZI1 (**f2**), haploid No. 858 (**f3**), No. 857 (**f4**). *Bar* 10 cm

Table 2 Morphology of the haploid plants from cross Hua24 × HZI1

Morphological traits	Haploid			Hua24			*t* value	Pr > |*t*|
	Mean	SE	SD	Mean	SE	SD		
Plant height (cm)	114.14	2.31	10.59	175.38	2.81	13.78	−16.82	<0.0001
Ear height (cm)	39.05	1.90	8.70	88.88	1.95	9.56	−18.31	<0.0001
Ear leaf length (cm)	55.94	1.11	5.08	77.08	0.98	4.80	−14.29	<0.0001
Ear leaf width (cm)	6.37	0.19	0.87	8.85	0.13	0.62	−10.85	<0.0001
Tassel length (cm)	19.73	0.70	3.19	30.42	0.58	2.84	−11.78	<0.0001
Tassel branches	19.95	1.18	5.40	23.79	0.81	3.97	−2.69	0.0108

SD standard deviation, *SE* standard error

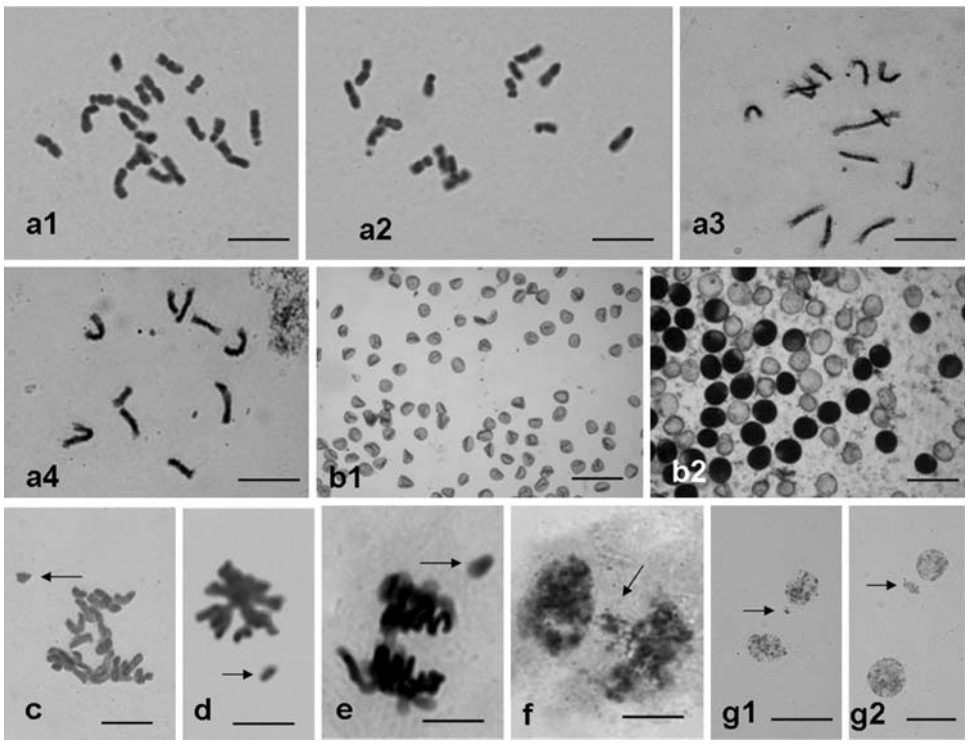

Fig. 2 Cytology of F_1 kernels from Hua24 × HZI1 cross. **a1**–**a4** Root-tip metaphases in the radicles of the F_1 kernels, with $2n = 20$ (**a1**), 15 (**a2**), 13 (**a3**), 10 (**a4**). *Bar* 10 μm. **c** One lagged chromosome in root metaphase. **d**–**g** Ovule cells of Hua24 pollinated by HZI1. *Bar* 10 μm. **d** One chromosome lagged at metaphase. **e** One chromosome lagged at anaphase. **f** Laggards at telophase. **g1**, **g2** Micronuclei at telophases. **b1**, **b2** Pollen grains of haploids Nos. 869, 867. *Bar* 20 μm

Chromosome numbers in the female parents HZ85 and Hua24 and inducer HZI1

In radicles of normal inbred line HZ85, 96.70% cells had the expected chromosome numbers $2n = 20$ and 3.30% had $2n < 20$ (Table 3). In Hua24, 96.85% cells had $2n = 20$ and 3.15% had $2n < 20$ (Table 3). For inducer HZI1, only 85.41% cells had $2n = 20$ and 14.59% had $2n < 20$ but 9.75% had $2n = 10$, the number of haploid cells. Among the radicles of HZI1, 25% had $2n = 20$ in all cells, 10% had $2n = 10$ in majority of cells, and 65% had $2n = 20$ as most frequent in their cells. So HZI1 can produce haploids in the selfed progeny.

Cytology of F_1 radicles and pollinated ovules

In the radicles of F_1 kernels with purple aleurone and colorless embryo and F_1 kernels with purple aleurone and purple embryos obtained from the crosses Hua24 × HZI1, HZ124b × HZI1, HZ141 × HZI1, and HZ85 × HZI1, the cells with different chromosome numbers ($2n = 9$–21) were observed, with $2n = 10$, or 20 being most frequent in different roots (Table 4). In the radicles of F_1 kernels with purple aleurone and colorless embryo, the frequencies of cells with $2n = 10$ were 67.47, 76.74 and 45.22% in crosses of HZI1 with Hua24, HZ124b, and HZ141, respectively. But in the cross HZ85 × HZI1, the frequency

Table 3 Different chromosome numbers in the radicle cells of cross parents

Lines	Cells with chromosome numbers											Total
	10	11	12	13	14	15	16	17	18	19	20	
HZ85												
Cell no.	0	1	2	0	0	1	0	1	2	1	343	351
(%)	0.00	0.29	0.57	0.00	0.00	0.29	0.00	0.29	0.57	0.29	96.70	
Hua24												
Cell no.	0	0	0	0	0	0	1	1	1	2	150	155
(%)	0.00	0.00	0.00	0.00	0.00	0.00	0.63	0.63	0.63	1.26	96.85	
HZI1												
Cell no.	51	2	2	2	4	2	5	1	2	5	447	523
(%)	9.75	0.38	0.38	0.38	0.76	0.38	0.98	0.19	0.38	0.98	85.41	

Table 4 Numbers and percentages of radicle cells in two types of embryos of F_1 kernels from four crosses

Crosses	Radicles		Number and percentage of cells with chromosome number													Total
			9	10	11	12	13	14	15	16	17	18	19	20	21	
Hua24 × HZI1	I	Number	8	253	49	10	22	4	0	4	2	5	1	17	0	375
		(%)	2.13	67.47	13.07	2.67	5.87	1.07	0.00	1.07	0.53	1.33	0.27	4.53	0.00	
	II	Number	7	22	13	8	5	4	8	12	4	13	9	127	2	234
		(%)	2.99	9.40	5.56	3.42	2.14	1.71	3.42	5.13	1.71	5.56	3.85	54.27	0.85	
HZ124b × HZI1	I	Number	5	165	15	5	4	3	2	0	2	2	2	10	0	215
		(%)	2.33	76.74	6.98	2.33	1.86	1.40	0.93	0.00	0.93	0.93	0.93	4.65	0.00	
	II	Number	2	5	4	4	4	4	7	7	7	7	12	123	2	188
		(%)	1.06	2.66	2.13	2.13	2.13	2.13	3.72	3.72	3.72	3.72	6.38	65.43	1.06	
HZ141 × HZI1	I	Number	29	194	13	8	19	4	5	5	6	13	21	111	5	429
		(%)	6.8	45.22	3.03	1.86	4.43	0.93	1.17	1.17	1.39	3.03	4.89	25.87	1.17	
	II	Number	4	8	6	6	5	8	5	14	9	13	26	139	5	248
		(%)	1.61	3.23	2.42	2.42	2.02	3.23	2.02	5.65	3.63	5.24	10.48	56.05	2.02	
HZ85 × HZI1	I	Number	18	193	17	7	6	15	15	16	16	31	24	329	4	691
		(%)	2.60	27.93	2.46	1.01	0.87	2.17	2.17	2.32	2.32	4.49	3.47	47.61	0.58	
	II	Number	12	27	14	15	12	17	22	17	22	47	69	391	7	672
		(%)	1.79	4.02	2.08	2.23	1.79	2.53	3.27	2.53	3.27	6.99	10.27	58.18	1.04	

I, Radicles of kernels with purple aleurone and colorless embryo; II, radicles of kernels with purple aleurone and purple embryo

of the cells with $2n = 10$ was 28%, while that of those with $2n = 20$ was 47.60%. In the radicles of F_1 kernels with purple aleurone and purple embryo from the four crosses, the cells with $2n = 20$ were 54.27, 65.43, 56.05, and 58%, respectively.

More than half (56.4%) of the radicles of F_1 kernels with purple aleurone and colorless embryo had variable chromosome numbers ($2n = 9$–21) and the kernels were classified into 6 categories: I, haploid, $2n = 10$ in all cells; II, mixoploid haploid, $2n = 10$–15 with $2n = 10$ of most frequent; III, mixoploid haploid, $2n = 10$–20 with $2n = 10$ of most frequent; IV, mixoploid diploid, $2n = 10$–20 with $2n = 20$ of most frequent; V, mixoploid, $2n = 11$–19; VI, diploid, with $2n = 20$ in all cells (Table 5). Types I and II were more frequent in crosses Hua24 × HZI1, HZ124b × HZI1, while types II and IV in crosses HZ141 × HZI1, HZ85 × HZI1. In crosses Hua24 × HZI1, HZ124b × HZI1, percentages of haploid radicles were 45 and 32%, respectively, but only 16.67 and 2.23% in crosses HZ141 × HZI1, HZ85 × HZI1.

The kernels with purple aleurone and purple embryo had 62.5% radicles with different chromosome numbers ($2n = 9$–21) and were put into five types: I, diploid, $2n = 20$ only; II, mixoploid diploid, $2n = 10$–20 with $2n = 20$ being most frequent; III, mixoploid diploid, $2n = 16$–20 with $2n = 20$ being most frequent; IV, mixoploid haploid, $2n = 10$–20 with $2n = 10$ being most frequent; V, mixoploid, $2n = 11$–19 (Table 5). Types I, II and III were more frequent in all four crosses. The percentages of diploid kernels in four crosses were 37.5, 16.12, 20, and 12.5%, respectively (Table 5).

In ovule cells from the cross Hua24 × HZI1, individual chromosomes were arranged out of equatorial plate in metaphase cell (Fig. 2d) and lagged at anaphase/telophase cells (Fig. 2e, f). Micronuclei were formed (Fig. 2g1, g2).

Change of cells with different chromosome numbers in radicles at three times

In the radicles of the kernels with purple aleurone and colorless embryos from the cross HZ85 × HZI1 collected 3, 5, 7 days after germination, the frequencies of the cells with $2n = 10$ increased from 13.29, to 53.88, to 70%, while those of $2n = 20$ decreased from 65.19, to 20.87, to 10% (Fig. 3a). In the radicles of the kernels with purple aleurone and purple embryos at three times, the frequencies of the cells with $2n = 10$ and 20 varied at much narrower ranges and cells with $2n = 20$ were predominant (about 50%); the percentages of $2n = 20$ are 59.32, 57.32, 48.13%, respectively; and those of $2n = 10$ are 5.08, 1.22, 5.00%, respectively (Fig. 3b).

SSR analysis

SSR analysis using 28 markers on haploid plants from the cross Hua24 × HZI1 showed that all haploids shared the same genomic compositions as the female Hua24 except two (Nos. 857 and 862) (Fig. 4a, b). Haploid No. 857 plant

Table 5 Numbers and frequencies of the kernels with different chromosome numbers in two types of embryos from four crosses

Crosses	Phenotype	Type	Most frequent one and ranges of chromosome numbers	Plant number	(%)	Total
Hua24 × HZI1	i	I	10	18	45.00	40
		II	10;10–15	14	35.00	
		III	10;10–20	3	7.50	
		IV	20;10–20	2	5.00	
		V	11–19	3	7.50	
		VI	20	0	0.00	
	ii	I	20	15	37.50	40
		II	20;10–20	18	45.00	
		III	20;16–20	5	12.50	
		IV	10;10–20	1	2.50	
		V	11–19	1	2.50	
HZ124b × HZI1	i	I	10	8	32.00	25
		II	10;10–15	14	56.00	
		III	10;10–20	0	0.00	
		IV	20;10–20	2	8.00	
		V	11–19	0	0.00	
		VI	20	1	4.00	
	ii	I	20	5	16.12	31
		II	20;10–20	13	41.93	
		III	20;16–20	13	41.93	
		IV	10;10–20	0	0.00	
		V	11–19	0	0.00	
HZ141 × HZI1	i	I	10	6	16.67	36
		II	10;10–15	11	30.56	
		III	10;10–20	2	5.56	
		IV	20;10–20	13	36.11	
		V	11–19	2	5.56	
		VI	20	2	5.56	
	ii	I	20	6	20.00	30
		II	20;10–20	17	56.67	
		III	20;16–20	5	16.67	
		IV	10;10–20	0	0.00	
		V	11–19	2	6.67	
HZ85 × HZI1	i	I	10	1	2.63	38
		II	10;10–15	6	15.79	
		III	10;10–20	1	6.67	
		IV	20;10–20	19	50.00	
		V	11–19	7	18.42	
		VI	20	4	10.53	
	ii	I	20	7	12.50	56
		II	20;10–20	40	71.43	
		III	20;16–20	7	12.50	
		IV	10;10–20	0	0.00	
		V	11–19	2	3.58	

i, Radicles of kernels with purple aleurone and colorless embryo

ii, Radicles of kernels with purple aleurone and purple embryo

had 61% heterozygous bands, 7.1% from maternal parent, 3.6% from paternal parent and 3.6% novel for two parents. Haploid No. 862 had 43% loci novel for two parents, 30% from maternal parent and 15% from paternal parent. The SSR clustering of the haploid plants selected by morphology from the cross Hua24 × HZI1 showed that all the

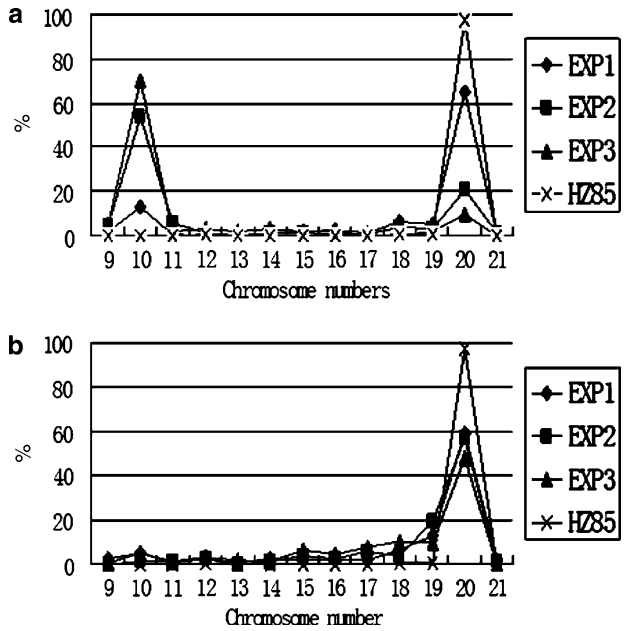

Fig. 3 Frequencies of radicle cells with different chromosomes in F_1 kernels from the cross HZ85 × HZI1 at three times. **a** Kernels with purple aleurone and colorless embryos. **b** Kernels with purple aleurone and purple embryo. EXP1, EXP1, and EXP3: radicles collected 3 days (EXP1), 5 days (EXP2), 7 days (EXP3) after generation, respectively

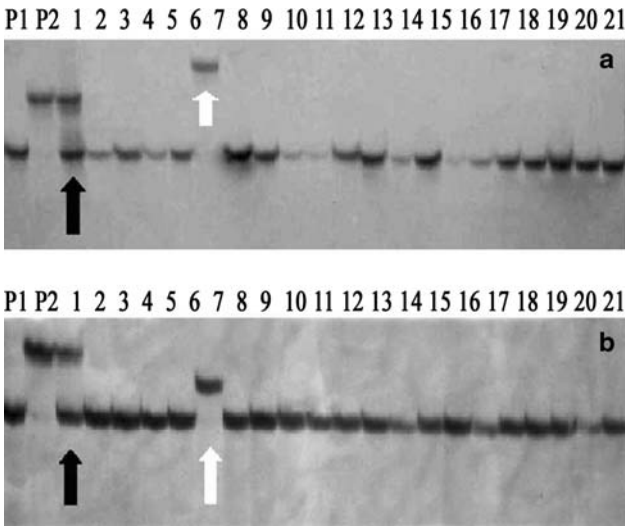

Fig. 4 Representative SSR profiles generated from two markers in the haploid plants from cross Hua24 × HZI1. **a** From primer M178 (umc2059). Black arrow shows heterozygous band of haploid No. 857, and white arrow shows one novel band in haploid No. 862. **b** From primer M219 (bnlg1065). *Black arrow* shows heterozygous band in haploid No. 857, and *white arrow* shows novel band in haploid No. 862. *P1* Hua24, *P2* HZI1

haploid plants were put between the two parents and most haploids were close to the female parent Hua24 except for plants Nos. 862 and 857 (Fig. 5).

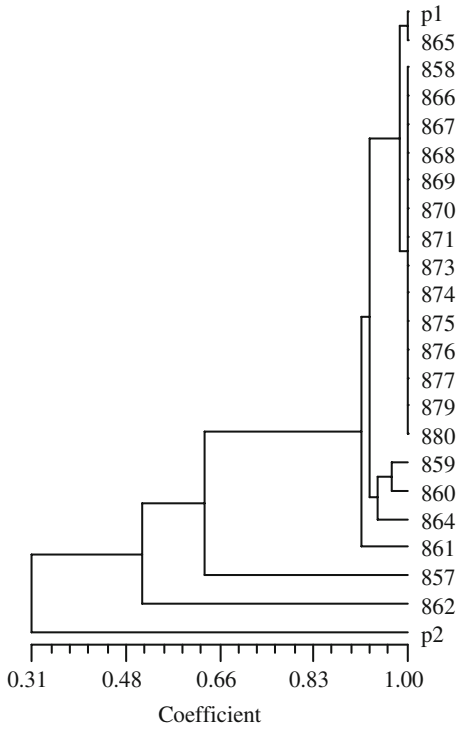

Fig. 5 SSR clustering of haploid plants from Hua24 × HZI1 cross. *p1* Hua24, *p2* HZI1

Twenty-five plants from the cross HZ124b × HZI1 showed the same SSR profiles and shared the same genomic compositions as the female HZ124b.

Discussion

The frequencies of haploid plants from the kernels with purple aleurone and colorless embryo from the four crosses varied widely from 25.09 to 88.0% (Table 5), as reported by others (Lashermes et al. 1988; Shatskaya et al. 1994; Chalyk 1999; Eder and Chalyk 2002). Progenies from inducing hybridizations with haploid inducer were investigated for morphology, cytology and genomic compositions, which enable us to identify the haploids efficiently. Most putative haploids screened by *R-nj* morphological marker were similar with the maternal parent in phenotype. Cytology revealed that some lagged chromosomes and micronuclei occurred in the mitotic cells of fertilized ovules (Fig. 2) and mixoploidy in the radicle of the F_1 kernels (Tables 4, 5; Fig. 2). Some paternal bands were found in few haploids (Nos. 857 and 862) by SSR analysis (Figs. 4, 5). The kernels with mosaic endosperm were found in the cross Hua24 × HZI1 (Fig. 1).

Lagged chromosomes (Fig. 2d, e, f) and the micronuclei (Fig. 2g1, g2) were often used as direct evidence of chromosome elimination and haploid production (Kasha and

Kao 1970), which often occurred in inter-, intra-specific hybridizations in *Gramineae* and other species (Laurie 1989). Micronuclei were also found in the primordium of the inducer RWS ovaries 20 days after self-pollination (Wedzony et al. 2002). Hence, the results indicate that the chromosome elimination during the early development of the embryo after fertilized by inducers is one of the main causes of the in vivo haploid induction in maize.

About 15% radicle cells of the inducer HZI1 were aneuploid, while about 3% in inbred lines. Similar results were also observed for microsporocytes of the inducer MHI and two inbred lines used as checks (Chalyk et al. 2003). So aneuploidy is involved in haploid induction by the inducer and occurred in the microsporocytes and radicle cells of inducers, which showed that the chromosomes of the inducer were prone to lose during mitotic and meiotic divisions and provided the cytological basis for the induction ability to produce haploids in crosses with other lines.

Among six types of F_1 kernels with purple aleurone and colorless embryos in four crosses, Types I, II, III were haploid plants in the field, showing that the chromosomes from HZI1 were completely lost during the development process of embryos and plants. In F_1 radicles collected at different times, the frequency of cells ($2n = 10$) gradually increased, while that of the cells ($2n = 20$) reduced. Radicles of putative haploid kernels also had some cells with $2n = 20$ (Tables 4, 5), but the frequency of cells with $2n = 10$ tended to increase (Fig. 3a), leading to the production of haploid plants. This also indicated that the chromosome elimination probably occurred in the whole growth process of the haploid plants.

Diploid cells in the maize haploid tissues were likely due to the doubling of haploid cells (Khokhlov et al. 1976) or the residue of the chromosome elimination. Plants of types IV, V looked like hybrid plants in the field probably for the high percentages of diploid cells in apical meristem of plants, which was known as incorrect selection by the expression of the *R-nj* gene (Beckert 1994). Kernels of type VI probably did not look like haploids or hybrids, as found by Chalyk et al. (2003). All haploid plants were smaller than the diploid parents (Rotarenco 2000) had low pollen fertility (Shatskaya et al. 1994). Diploid cells in haploid No. 867 of type III were probably from the doubling of haploid cells and resulted in higher pollen stainability (Fig. 2b2). The female spikelet of the haploid plants from the cross Hua24 × HZI1 developed abnormally and had lower seed-sets than that of others (Chalyk 1994), which was possibly affected by the environment and cultivation conditions.

Of five types of kernels with purple aleurone and purple embryo in all four crosses, Types I, II, III were phenotypically hybrid plants, but variations in chromosome numbers were shown by Types II, III. Types IV and V often presented some morphological deviations from hybrid plants, as found by Chalyk et al. (2003), which might be related with their wide ranges of chromosome numbers in cells. The mixoploidy nature of these plants should be the result of partial elimination of chromosomes from HZI1. The morphological traits of haploid No. 857 are different from that of other haploids and female parent Hua24, and some SSR DNA bands from HZI1 indicated the introgression of genetic element from the inducer during the process of chromosome lost, the same results was found by Fischer (2004). Haploid No. 862 with the same morphology as other haploids had 43% loci novel for two parents, which indicated that No. 862 was not caused by the pollen contamination and the high rate of the novel bands might be attributable to the heterozygosity of HZI1. In mixoploid No. 862 ($2n = 9$–21) with high frequency of cells ($2n = 10$) and SSR loci (15%) from the inducer the incomplete chromosomes elimination with some chromosomal rearrangements probably occurred. The SSR and morphology clusterings also separated plants Nos. 862 and 857 from all other haploids (Fig. 5). But the maternal haploids from another cross HZ124b × HZI1 in morphology and genomic compositions were probably either due to the genotypic effect of different female parents.

In about 0.2% kernels of F_1 progeny from the cross Hua24 × HZI1, endosperms were mosaic (Fig. 1c, d1, d2, d3), while F_1 hybrid kernels should have normal endosperm with purple aleurone. According to the genotypes of two parents, the mosaic kernels most likely resulted from the elimination of chromosomes carrying *R-nj* and *Sh2*, because the rate of simultaneous mutation of two genes is very slow [the mutation rates of the *R-nj* and *Sh2* genes in maize kernels are about 5×10^{-4} and 1.2×10^{-6} (Zhu 2002), respectively, and the rate of transpositional insertion is about 10^{-5} (Zhu 2002)]. The variable sizes of the shrunken sweet parts (Fig. 1c, d1, d2, d3) in the mosaic endosperm may be attributed to the different elimination times of paternal chromosomes. The mosaic kernels (Fig. 1c) were probably the result of the complete elimination in one daughter cell from the first mitosis of the primary endosperm nuclei. The mosaic kernels in Fig. 1d1, d2, d3 formed later and the sweet shrunken endosperm often occurred at the top of the endosperm.

Complete and partial elimination of chromosomes from one parent was documented in plant wide crosses (Kasha and Kao 1970; Laurie 1989; Laurie and Bennett 1989; Mochida et al. 2004; Riera-Lizarazu et al. 1996; Gernand et al. 2005, 2006). Chromosome elimination was generally attributed to the unbalanced chromosome sets (Kasha and Kao 1970). The chromosome elimination mechanism behind in vivo haploid induction in maize may be similar to that of wide hybridizations or be caused by some genetic factors in the inducers (Lashermes and Beckert 1988; Deimling et al. 1997; Barret et al. 2008), which need to

elucidate in future and may be important for the selection of inducers with high frequency.

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