Planta (2009) 230:367–376
DOI 10.1007/s00425-009-0943-1

ORIGINAL ARTICLE

Morphological and molecular evidences for DNA introgression in haploid induction via a high oil inducer CAUHOI in maize

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Received: 12 March 2009 / Accepted: 27 April 2009 / Published online: 24 May 2009

Abstract The phenomenon of maternal haploid induction in maize was first described many years ago, but the underlying mechanism is still unclear. In this study, the Stock-6-derived, haploid-inducing line CAUHOI with high kernel oil content (KOC), was used as the pollinator to produce maternal haploids from the maize hybrid ZD958 with low KOC. CAUHOI is homozygous for the dominant marker gene *R1-nj*. Haploids were identified by morphological and cytological investigations. The frequency of haploid induction from this cross was 2.21%. Unexpectedly, many haploid kernels had weakly pigmented purple color on the embryo, and some haploid kernels had high KOC. Simple sequence repeat (SSR) analysis showed that 43.18% of the haploids carried segments from CAUHOI, and a small proportion (average 1.84%) of the genome of CAUHOI was introgressed into haploids. Haploid kernels with high KOC had a higher frequency of segment introgression from CAUHOI (2.92%) than that in haploid kernels with low KOC (1.79%), showing that the marker gene *R1-nj* and high-oil genes from CAUHOI were expressed during the development of some haploid embryos, and confirmed that the DNA introgression from the inducer parent occurred during maternal haploid induction. Together, these results suggested that the chromosome elimination was probably responsible for haploid induction in maize, and late somatic elimination might occur. Several possible mechanisms underlying haploid formation are discussed.

Keywords Chromosome elimination · DNA introgression · Haploid · Maize · Oil · Simple sequence repeat

Abbreviations

DAPI	4′-6-Diamidino-2-phenylindole
DH	Double haploid
KOC	Kernel oil content
NMR	Nuclear magnetic resonance
QTL	Quantitative trait locus
SSR	Simple sequence repeat

Introduction

Double fertilization in flowering plants is one of the defining features of reproductive development. It is known that one sperm fuses with the egg cell, which develops into an embryo, and the other sperm fuses with the central cell, which develops into an endosperm; however, the underlying mechanism is not well understood. Several female gametophyte mutants, including *feronia* (Huck et al. 2003), *sirene* (Rotman et al. 2003), and *ig1 (indeterminate gametophyte1)* (Evans 2007) have been studied in attempts to elucidate the molecular mechanism involved in double fertilization. Moreover, an Arabidopsis mutant (*CDKA; 1*) with one sperm cell in the pollen grain has been described (Nowack et al. 2006). Despite these findings, the exact

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role(s) of the plant male gametophyte in the double fertilization process is unclear (Barret et al. 2008). Study of the plant male gametophyte leading to gynogenesis will provide more information about the double fertilization process.

Gynogenesis in plants is defined as a form of asexual reproduction, which has the ability to produce an embryo originating exclusively from the egg cell. This feature has been studied in vivo for *Hordeum vulgare* (Hagberg and Hagberg 1980), and in vitro for *Allium cepa* (Martínez et al. 2000) and for *Cucumis sativus* (Gémes-Juhász et al. 2002). Gynogenesis was also obtained following wide hybridization crosses through chromosome elimination (Subrahmanyam and Kasha 1973; Gernand et al. 2005). Normally, gynogenesis is stimulated by the presence of sperm cell. In maize (*Zea mays* L.), some genotypes produce pollen that is able to induce maternal haploids (Chase 1949). Coe (1959) first reported the maternal haploid inducer Stock-6, with an induction rate of about 1%. The haploid-inducing capacity of the inducer can be increased by selection (Sarkar et al. 1972), and the new inducers with higher rates of haploid induction have been obtained, such as WS14 (Lashermes and Beckert 1988), ZMS (Chalyk 1994), MHI (Eder and Chalyk 2002), and RWS (Röber et al. 2005).

These haploid inducers in maize are widely used to produce double haploid (DH) lines because of the tremendous advantages in comparison to the methods used in conventional breeding programs (Seitz 2005), but they can also be considered as original mutant lines for the analysis of double-fertilization mechanisms. Bylich and Chalyk (1996) observed that 6.32% of pollen grains had two sperm nuclei of different sizes in the inducer ZWS, whereas Mahendru and Sarkar (2000) found no difference in size between the two sperms in a maize-inducing line. Liu and Song (2000) suggested that the abnormal pollen tubes, with two spatially well-separated sperm nuclei, might be the reason for haploid induction. Rotarenco and Eder (2003) detected a much higher rate of heterofertilization when the haploid inducer MHI was used compared to a normal line. Chalyk et al. (2003) detected up to 15% aneuploid microsporocyte in the inducer line and only about 1% in two inbred lines used as controls. All 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 embryo can be formed from an unfertilized egg cell; however, Fischer (2004) observed paternal DNA transmission in a small proportion of haploids. Micronuclei, generally accepted as evidence of chromosome elimination (Kasha and Kao 1970), have been found during maternal haploid induction in maize (Wedzony et al. 2002; Zhang et al. 2008), but these cannot be interpreted by single fertilization with the central cell. Despite all the advances, whether the formation of female haploid embryos results from single fertilization or from chromosome elimination remains unclear.

In this study, the Stock-6-derived inducing line CAUHOI with high kernel oil content (KOC) was used to induce haploids from the maize single-cross ZD958 with low KOC. The main objectives were: (1) to report the morphological observation and KOC measurements of the haploids; (2) to discover whether DNA introgression from the inducer parent into the genome of maternal haploid plants occurred during maternal haploid induction; and (3) to provide insight into the fundamental biological mechanism of maternal haploid induction in maize.

Materials and methods

Haploid induction

CAUHOI, a Stock-6-derived haploid-inducing maize line with high KOC (Chen and Song 2003) was used as the male parent. The maize single-cross ZD958, developed by the Henan Academy of Agriculture Science (Henan Province, China), was used as the female parent. The cross was made manually at the Changping Experimental Station in Beijing in 2007, and a total of 25,000 kernels were harvested from 85 ears.

Kernel classification and KOC measurement

Line CAUHOI is homozygous for the dominant marker gene *R1-n j* (Nanda and Chase 1966), leading to a purple scutellum and a "purple crown" aleurone of F1 kernels when crossed with unpigmented donors. We used these two characteristics as embryo and endosperm markers, respectively. Normally, the kernels with a haploid embryo and a regular triploid endosperm display colorless embryos and purple endosperms, whereas the hybrid kernels with a diploid embryo show purple pigmentation on both embryo and endosperm. A new kind of kernel was detected from the cross ZD958 × CAUHOI, whose purple pigmentation on the embryo was clearly different from the above-mentioned haploid and hybrid kernels, and we named them diploid-like kernels. To study this new kind of kernel, the harvested kernels were classified into three groups: (1) putative haploids, kernels with purple on the endosperm and no purple on the embryo; (2) hybrid kernels, kernels with purple on the endosperm and strongly pigmented purple on the embryo; (3) diploid-like kernels have purple on the endosperm and weakly pigmented purple on the embryo. All the putative haploids, diploid-like kernels, and 2,000 hybrid kernels chosen at random were

used to measure the KOC with nuclear magnetic resonance (NMR) (Bauman et al. 1963; Conway and Johnson 1969); KOC was also measured for 100 selfed kernels of ZD958 and CUAHOI.

Determination of ploidy level and morphological observation

Line CAUHOI also carries a dominant purple-stem marker. Thus, the ploidy levels of the plants were first screened by stem color, plant height and male fertility, and then determined by chromosome counting. Chromosomal preparation was made as described by Mukai et al. (1990), and the enzyme-squash and 4′-6-diamidino-2-phenylindole (DAPI) staining followed the methods reported by Moscone et al. (1996). After staining with DAPI, the chromosomes were viewed with a Leitz Laborlux-S fluorescence microscope equipped with a Mono-Cool View CCD camera. The chromosome number in each root tip was determined as described by Ramanna and Hermsen (1971). Morphological characters, such as plant height, ear height, stem color, and leaf color, were measured at 20 days after flowering. After determination of the ploidy level, the haploid kernels from the putative haploid group and the diploid-like kernels group were renamed as normal haploids and diploid-like haploids, respectively; and the diploids from the diploid-like kernels group were simplified as diploid-like diploids.

Simple sequence repeat (SSR) marker and sequence analysis

A total of 26 normal haploid plants and 62 diploid-like haploid plants were chosen at random for SSR analysis. Maize genomic DNA was extracted by the CTAB procedure (Saghai-Maroof et al. 1984) and, in total, 300 SSR markers were screened for polymorphisms between ZD958 and CAUHOI. Finally, 40 SSR markers with clear polymorphisms were used, which covered all 10 chromosomes, and all SSR markers were obtained from the MaizeGDB database (http://www.maizegdb.org/ssr.php). DNA amplification and polymorphism identification were performed as described by Ninamango-Cárdenas et al. (2003). All of the 19 diploid-like haploids with high KOC (>48.40 g/kg, where 48.40 g/kg was the highest value of KOC of selfed kernels from ZD958) and 10 randomly chosen diploid-like haploids with low KOC were sequenced on the core region of gene *DGAT1-2* by the two primers with accession numbers No. CS727014 and No. CS727015, respectively. *DGAT1-2* is a major high oil gene reported by Zheng et al. (2008), in which insertion of phenylalanine (TTC) at position 469 is responsible for the increased contents of oil and oleic acid. The sequences of the haploids were compared with ZD958 and CAUHOI.

Results

Purple pigmentation and frequency of haploids

The marker gene *R1-nj* was well expressed in the cross ZD958 × CAUHOI (Fig. 1a), and the kernels were classified into three groups (Fig. 1b). The diploid-like kernels had weak pigmentation on the embryo (Fig. 1b2), which was clearly different from the hybrid kernels (Fig. 1b1) and the putative haploids (Fig. 1b3). The frequency of diploid-like kernels and putative haploids was 1.42 and 1.25%, respectively (Table 1). Up to 78.03% of the diploid-like kernels were finally determined as diploid-like haploids, 87.86% of the putative haploid kernels were confirmed as normal haploids, and no haploid was detected in the kernels sampled from the hybrid kernels group. The haploid induction rate was 2.21% in the cross ZD958 × CAUHOI, including 1.10% normal haploids and 1.11% diploid-like haploids.

Variation of KOC among diploid-like kernels

The average KOC of CAUHOI (78.05 g/kg) was more than twice that of ZD958 (35.42 g/kg). All the normal haploids had low KOC with a mean value of 33.25 g/kg, which was a little lower than that of the selfed kernels of ZD958. Among the 277 diploid-like haploids, most kernels had a low KOC similar to that of normal haploids; however, 19 kernels with high KOC (> 48.40 g/kg) were detected. All the diploid-like diploids had KOC >48.40 g/kg. As a whole, the average KOC of all the haploids, including the diploid-like haploids, was 37.50 g/kg, significantly lower than the average KOC (60.03 g/kg) of hybrid kernels (Fig. 2).

Morphology and cytology of the diploid-like plants

From seedling to maturity, no difference of morphological performance was detected between normal haploid plants and diploid-like haploid plants. Compared to the diploids, the haploid seedlings were shorter, with slender weak stems and narrower leaves, and grew more slowly (Fig. 1d). When flowering, except some haploids showed partial fertility in some branches or florets (Fig. 1e), most haploids were male-sterile (Fig. 1f). The haploid plants had green stems and leaves. The diploid-like diploid plants showed the same performance of purple stems and leaves as hybrid plants. All root tips showed euploid chromosomes ($n = 10$ or 20) (Fig. 3a–c), and no lagging asymmetric inducer chromosome was detected in the hybrid root tip cells (Fig. 3d).

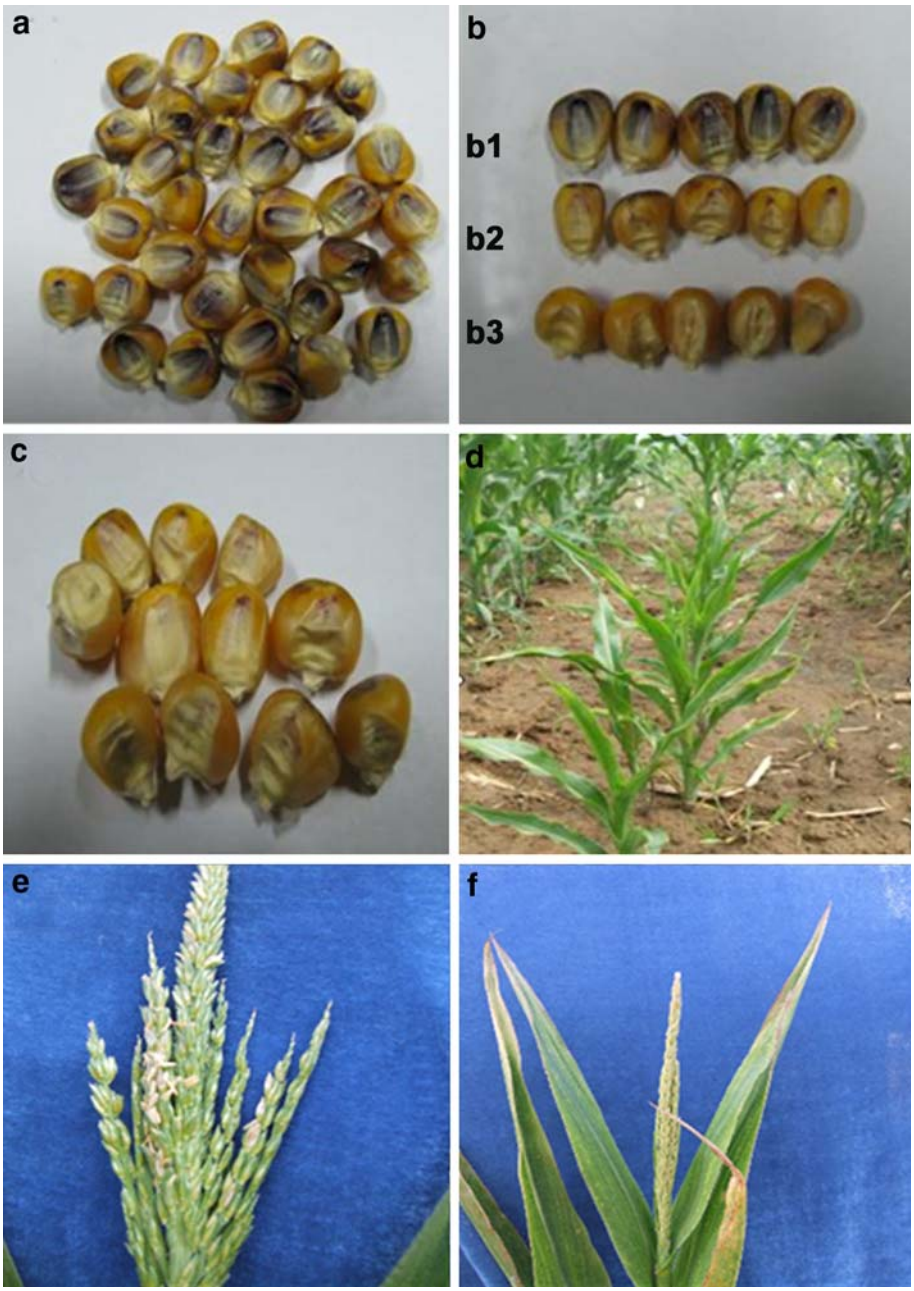

Fig. 1 Morphology of the progeny from the cross ZD958 × CAUHOI. **a**–**c** F1 kernels from the cross ZD958 × CAUHOI. **a** Hybrid kernels with strong purple pigmentation on the embryo. **b** Three types of F1 kernels, **b1**, hybrid kernels; **b2**, diploid-like kernels; **b3**, putative haploids with colorless embryo. **c** Diploid-like kernels with weak purple pigmentation on the embryo. **d** Field performance of diploid-like haploid seedlings; **e** Haploid tassel with partial fertility. **f** Haploid tassel with complete male sterility

The frequency of DNA introgression from CAUHOI into the haploids

Among the 88 haploids (including 62 diploid-like haploids), there were 38 carrying segments from CAUHOI (Fig. 4a, b), at a frequency up to 43.18%. Most of the haploids carried segments from CAUHOI with a frequency between 0 and 17.50%, except one diploid-like haploid (No. 48) with a frequency of 58.97%. Haploid No. 48 had 41.03% loci from the maternal parent, 43.59% loci with heterozygous bands, and 15.38% loci from the paternal parent. Heterozygous bands were observed in other haploids with relatively low frequency.

Neglecting haploid No. 48, 64 segments from CAUHOI were detected in the haploids, and the frequency of segment introgression was 1.84%, on average, of all the detected sites. Among the 64 loci, 31 had heterozygous bands and 33 had paternal bands. The introgression frequency varied a great deal among different markers. The highest frequency (8.05%) was for 3 markers at locus 2.06 (p-nc003) and 6.04 (umc2006 and umc1014), which meant that at each locus there were seven haploids that carried a segment from CAUHOI. There were also 14 markers (35% of detected markers) with no introgression into the haploid genome.

Table 1 The frequency of different types of kernels and the haploids determined from the cross ZD958 × CAUHOI

	Number of kernels	Frequency[a] (%)	Haploids determined	Rate of haploids[b] (%)	Induction rate[c] (%)
Putative haploids	313	1.25	275	87.86	1.10
Diploid-like kernels	355	1.42	277	78.03	1.11
Hybrid kernels	24,352	97.41	0	0.00	0.00

[a] Frequency of different kernel types compared to the total number of kernels

[b] Frequency of haploid kernels among each kernel type

[c] Frequency of haploid kernels in each kernel type among the total number of kernels

Different haploid types with different frequency of DNA introgression

The frequency of DNA introgression was widely varied among the haploids. In order to investigate the source of such variance, the diploid-like haploids (excluding No. 48) were classified into two groups according to the KOC value; high-oil haploids with KOC >48.40 g/kg and low-oil haploids with KOC ≤48.40 g/kg. The frequency of segment introgression from CAUHOI was compared between the two groups of kernels. The results showed that the frequency of segment introgression in the normal haploids (1.44%) was a little lower than that in the low-oil haploids (1.79%), but much lower than that in the high-oil haploids (2.92%) (Table 2).

Sequence analysis

Compared to ZD958, CAUHOI carried the three nucleotides (TTC) of phenylalanine insertion (Fig. 5), and haploid No. 18 was found to carry this phenylalanine insertion. However, the KOC of haploid No. 18 was 31.82 g/kg, lower than the average KOC of the selfed kernels of ZD958, as well as the normal haploids. None of the other haploids, including the 19 high-oil haploids, carried the three nucleotides TTC.

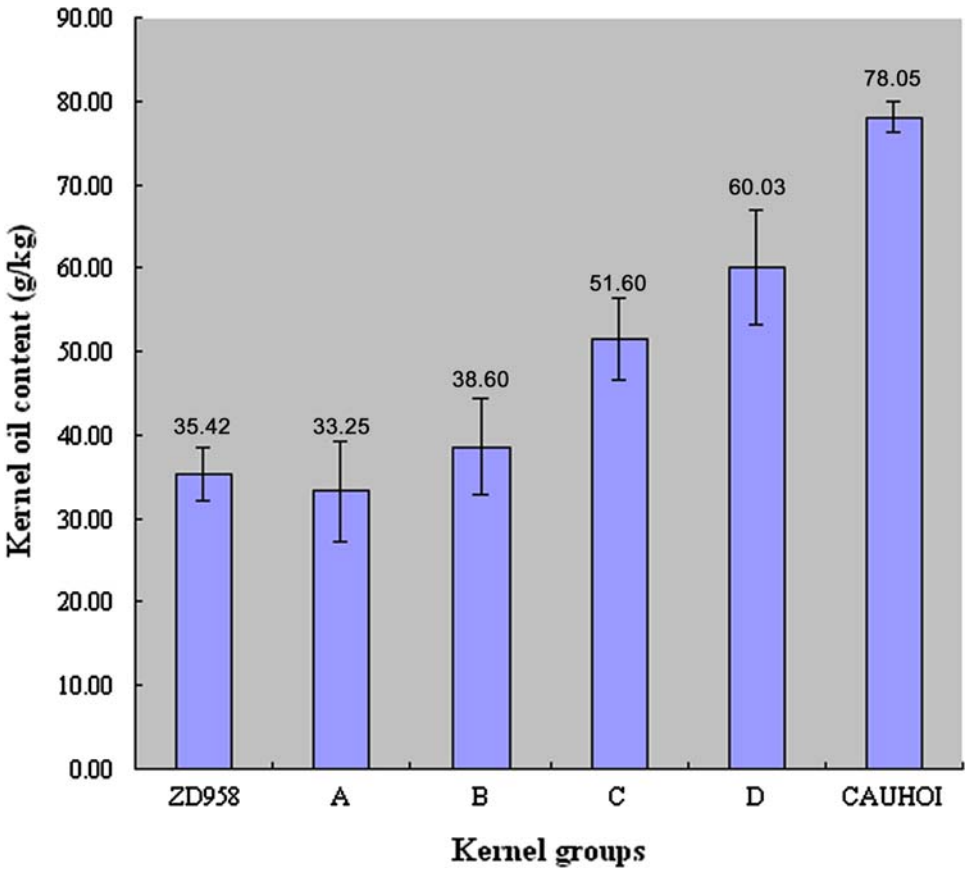

Fig. 2 Comparison of the oil content of different kernel types from the cross ZD958 × CAUHOI, with the selfed kernels of ZD958 and CAUHOI as controls. **a** Normal haploid kernels with colorless embryo. **b** Low-oil (≤48.4 g/kg) diploid-like haploids with weakly pigmented purple on the embryo. **c** High-oil (>48.4 g/kg) diploid-like haploids with weakly pigmented purple on the embryo. **d** Hybrid kernels

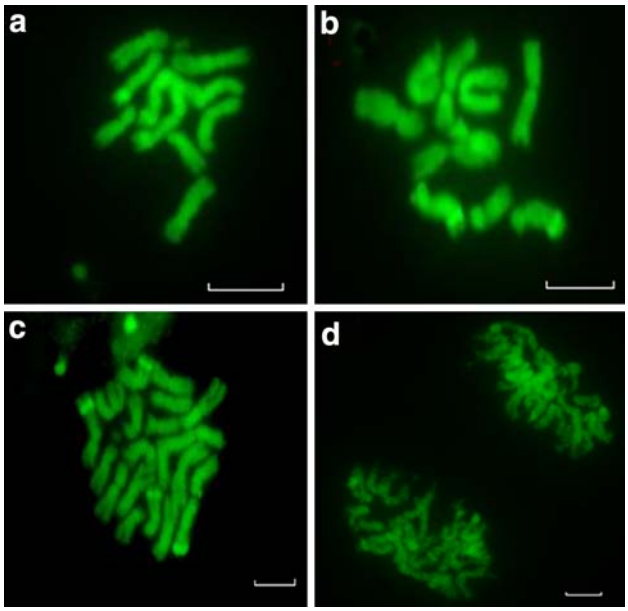

Fig. 3 Cytology of the root tips of the kernels from the cross ZD958 × CAUHOI. **a**, **b** Root tip cell with 10 chromosomes at metaphase. **c** Root tip cell with 20 chromosomes at metaphase. **d** Anaphase with no lagging asymmetric inducer chromosomes in the hybrid root tip cell. The *scale bar* represents 10 μm

Discussion

The marker gene *R1-nj* has been widely used for identification of haploid kernels from the cross by the Stock-6-derived inducing line (Nanda and Chase 1966; Chase 1969; Eder and Chalyk 2002). Normally, the kernels with purple on the embryo were first screened as hybrid kernels on the basis of the concept that the haploids were pure from the female parent (Lashermes and Beckert 1988; Chang 1992). Further investigations were conducted using mainly the putative haploids with a colorless embryo. In the cross ZD958 × CAUHOI, the expression of marker gene *R1-nj* was very clear, which enabled us to detect diploid-like haploid kernels ($n = 10$). These were named "diploid-like haploids", because they had weakly pigmented purple color on the embryo and looked like diploid kernels, but they were identified as haploids by chromosome counting. Additionally, CAUHOI is a high-oil line, and it has a strong xenia effect when it is used as the male parent (Chen and Song 2003). Most maize seed oil is located in the embryo (White and Weber 2003). Because the embryos of the hybrid kernels resulted from double fertilization of the egg cell from ZD958 with the sperm cell from CAUHOI, the KOC values of hybrid kernels were much higher than those of the selfed kernels of ZD958 (Fig. 2). If the embryos of the haploid kernels developed exclusively from the egg cell of ZD958, the KOC values of the haploid kernels should be similar to or lower than those of the selfed kernels of ZD958. However, in this cross, several diploid-like haploid kernels with high KOC, up to 60.00 g/kg, were unexpectedly detected, suggesting that the marker gene *R1-nj* and high-oil genes from CAUHOI were expressed in the development of some haploid embryos.

Using SSR markers, we detected 43.18% of the haploids carrying some segments from the paternal parent (CAUHOI). Even among the 26 normal haploids with a colorless embryo, only 57.69% (15) of haploids were observed with no segment from the paternal parent. Sequence analysis showed that haploid No. 18 carried the phenylalanine insertion (TTC) in the *DGAT1-2* gene from CAUHOI. These results confirmed that the DNA introgression from the inducer parent occurred during haploid induction in this cross. Nevertheless, the average frequency was 1.84% of all sites examined (Table 2), showing that only a small proportion of the inducer genome was introgressed into the haploids. This was in accordance with the morphology, in that no difference was detected between normal haploids and diploid-like haploids. DNA introgression in wide

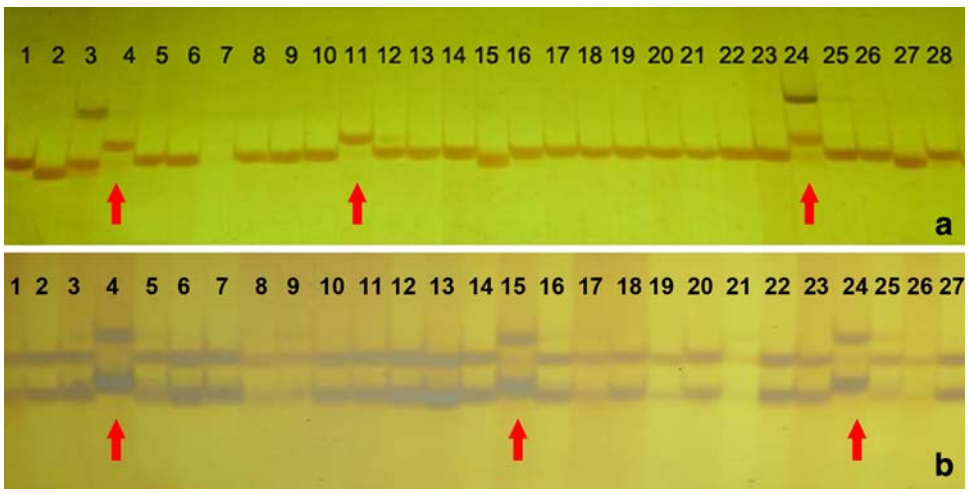

Fig. 4 Representative SSR profiles generated from two markers in the haploids from the cross ZD958 × CAUHOI. **a** From primer H318 (*bnlg2305*). **b** From primer H391 (*umc2127*). The *red arrow* indicates the novel band from CAUHOI

Table 2 Different frequencies of segment introgression from the paternal parent among different groups of haploid plants in the cross ZD958 × CAUHOI

Haploid type	No. haploids	Paternal sites	Paternal site frequency (%)	No. haploids without paternal site	Frequency of haploids with no paternal site (%)
Normal haploids	26	15	1.44	15	57.69
Diploid-like haploids[a]	49	35	1.79	32	65.31
Diploid-like haploids[b]	12	14	2.92	3	25.00
Sum	87	64		50	
Average			1.84		57.47

[a] Diploid-like haploids with low-oil kernels

[b] Diploid-like haploids with high-oil kernels

hybridization has been reported (Cui et al. 2009), but to our knowledge there is only one report of maternal haploid induction in maize. Fischer (2004) used SSR markers and observed that a small proportion (1–2%) of haploids carried one, and occasionally several, paternal chromosome segment. However, the frequency of haploids carrying paternal segments in that study was much lower than the frequency found in this study. The difference might come from the detection of diploid-like haploids or different research materials in our study. DNA introgression from the paternal parent cannot be explained by the hypothesis that a single sperm participated consequently in the single fertilization, indicating that the sperm cell from paternal parent may participate in fusion with the egg cell, and the chromosomes from paternal parent were eliminated in the subsequent divisions for some reason. Chromosome elimination and genetic introgression have been well documented in interspecific crossing of plants (Wilkinson et al. 1995; Pašakinskienė et al. 1997; Du et al. 2008). There is some evidence of chromosome elimination in maternal haploid induction in maize. Wedzony et al. (2002) studied the ovaries of the inducer RWS and found micronuclei of various sizes in the cytoplasm of every cell of the shoot tip primordium in about 10% of embryos. Zhang et al. (2008) found micronuclei in the ovary cell from the cross Hua24 × HZII. Micronuclei are often used as direct evidence of chromosome elimination and haploid production (Kasha and Kao 1970). These results suggested that the chromosome elimination is most likely one of the reasons for haploid induction via the Stock-6-derived inducing line in maize.

Let us re-examine the new phenomenon of high-oil haploid kernels found in this study. According to Liu et al. (2009), the oil was accumulated up to 58% at 15 days and up to 75% at 25 days after pollination. Compared to the hybrid kernels (with average KOC of 60.03 g/kg), those with KOC >48.40 g/kg implied that the oil was accumulated about 80%. There are two possibilities to explain this phenomenon: (1) the high-oil genes from CAUHOI were introgressed into the haploid genome; or (2) elimination of the chromosome from CAUHOI occurred after the accumulation of oil. However, KOC is a complicated trait that is controlled by several QTLs located on different chromosomes (Mangolin et al. 2004; Wassom et al. 2008). SSR marker analysis showed that only a small part of the CAUHOI genome was introgressed into haploids. So, there is small chance of introgression of all or most of high-oil genes at one event of haploid formation. This was also suggested by the sequence analysis, which showed that not all of the haploids with high KOC carry the phenylalanine insertion (TTC) in DGAT1-2 (Fig. 5). We found that the frequency of segment introgression from the paternal parent in the high-oil haploids was much higher than that in the low-oil haploids (Table 2). These results indicated that the late chromosome elimination might be responsible for the formation of haploid kernels with high KOC, and has been

Fig. 5 Comparison of the nucleotide sequence between the haploids and the parents CAUHOI and ZD958 on the gene *DGAT1-2* by the two primers with accession numbers No.CS727014 and No.CS727015. Haploid No. 5 and haploid No. 18 had low oil content; haploid No. 152 and haploid No.154 had high oil content

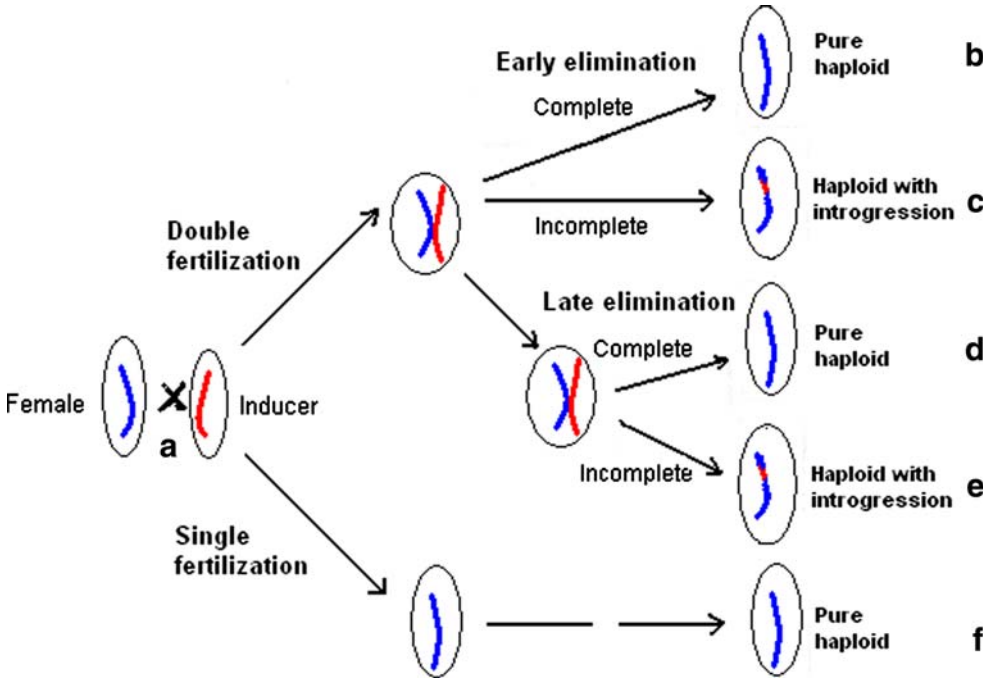

Fig. 6 Proposed model of maternal haploid induction in maize. Single fertilization and chromosome elimination can both lead to maternal haploid induction in maize. **a** Pollination with inducer as paternal parent. **b** and **d** Pure haploid with no paternal DNA introgression from complete chromosome elimination at early and late stages, respectively. **c** Haploids with some DNA introgression from incomplete chromosome elimination at the early stage. **e** Haploid with more DNA introgression from incomplete elimination at the late stage. **f** Pure haploid from single fertilization

reported to have increased potential of chromatin introgression into haploids (Gernand et al. 2005).

Among the 88 haploid plants, there were 50 that might be pure haploids because no paternal segment was detected by SSR analysis. Whether pure maternal haploids arise from complete chromosome elimination or by parthenogenesis could not be clarified with SSR markers. We think that chromosome elimination and single fertilization both may contribute to the haploid induction, and there are five possibilities for haploidization when chromosome elimination is involved (Fig. 6). (1) Pure haploids resulting from complete elimination during the very early stage; (2) haploids with paternal DNA introgression resulting from incomplete chromosome elimination during the early stage; (3) pure haploids resulting from complete chromosome during the late stage with paternal gene expression; (4) haploids with paternal DNA introgression resulting from incomplete elimination during the later stage, with more paternal gene expression and more genomic introgression; and (5) pure haploids resulting from single fertilization.

The morphological and molecular analysis of the progeny from the cross ZD958 × CAUHOI revealed that: (1) DNA introgression from CAUHOI occurred during haploid induction; (2) chromosome elimination may be responsible for haploid induction in maize; and (3) late chromosome elimination might occur during haploid induction. DNA introgression into haploids should be considered in future work because not all DH lines are genetically pure. Furthermore, it remains to be seen whether chromosome elimination or single fertilization, or both, represent the mechanism of haploid induction. Several hypotheses have been proposed to explain uniparental chromosome elimination in wide hybridizations (Subrahmanyam and Kasha 1973; Laurie and Bennett 1989; Kim et al. 2002; Mochida et al. 2004; Gernand et al. 2005). Nevertheless, the chromosome elimination in an intraspecific cross is rare, and the mechanism may be similar to that of wide hybridization or may be controlled by a specific gene. Complete analysis of the gynogenesis induction phenomena will provide new data for further investigation of double fertilization (Friedman 1998). Future work will need to make sure the chromosome elimination is responsible for the haploid induction by cytological investigation and determine the exact process of haploid development. This work will provide new information about the factors that affect double fertilization in flowering plants.

Acknowledgments This research was supported by funds from the National Natural Science Foundation of China (30370884, 30671306) and National 973 Project (2009CB118400). The authors greatly appreciate the helpful comments and suggestions from Prof. Dorothea Bartels and another anonymous reviewer. In addition, we thank Dr. C. Friedrich, H. Longin, and Dr. Thilo Wegenast for their valuable suggestions.

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