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Early spontaneous diploidization of maternal maize haploids generated by in vivo haploid induction

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Abstract The production of doubled haploid (DH) lines has become a key technology in maize (*Zea mays* L.) research and breeding. However, most of the haploid plants are sterile and in many cases artificial chromosome doubling involves the use of costly and toxic chemicals. Here, we report a special kind of doubled haploid named the early doubled haploid (EH) that was generated directly by in vivo haploid induction. We found 83 EH plants induced from the hybrid Zhengdan958, 55 families of its $F_{2:3}$ population and the parental lines, all of which were confirmed to be homozygous diploids via flow cytometry and 104 SSR markers. The progeny of EH_0 (EH_1) behaved in the same manner and showed the same potentialities as the parents of Zheng58 and Chang7-2. EH plants were also detected in other genetic backgrounds at a frequency of 1–3.5 % based on the total number of haploid plants. Because the EH lines exhibited completely fertility and were obtained from induction directly in one step, they could be used in DH breeding as a new breeding strategy. According to our observations, it is likely that spontaneous doubling in EH occurred during embryo development when haploid induction. The possible mechanism of EH is also discussed.

Keywords Maize · Chromosome doubling · Early doubled haploids · Haploid diploidization · Haploid induction

Abbreviations

DH	Doubled haploids
EH	Early doubled haploids
KOC	Kernel oil content
SSR	Simple sequence repeat

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Introduction

The development of homozygous inbred lines is important in the breeding of maize and many other crops. Breeders and researchers have traditionally produced inbred lines by selfing heterozygous materials for five to six generations, but this approach is expensive and time consuming (Eder and Chalyk 2002). Breeding with haploids can result in the creation of pure breeding lines (Szarejko and Forster 2007; Chang and Coe 2009; Geiger and Gordillo 2009) in fewer seasons.

Haploid induction and identification are not problematic with the selection of efficient haploid inducers, such as WS14 (Lashermes and Beckert 1988), ZMS (Chalyk 1994), MHI (Eder and Chalyk 2002), CAUHOI (Liu and Song 2000) and RWS (Röber et al. 2005). Haploids produced by these inducers are completely male-serile except for sectors in which doubling of chromosomes has taken place. There are two traditional methods for obtaining fertile haploids. One is spontaneous haploid doubling, for which doubling rates ranging from 0 to 21.4 % have been reported in various maize genotypes (Chase 1952; Chalyk 1994; Barnabas et al. 1999). The other is artificial chromosome doubling, which can duplicate the chromosome set of haploid plants effectively using doubling agents or physical stress during treatment. Many studies have focused on the improvement of artificial doubling efficiency (Stadler et al. 1989; Wan et al. 1991; Häntzschel and Weber 2010). Colchicine is the most widely applied artificial chromosome doubling agent (Gayen et al. 1994; Deimling et al. 1997). Despite the effectiveness of colchicine in artificial chromosome doubling and its suitability for large-scale DH line production (Gayen et al. 1994; Barnabas et al. 1999), in many cases its high cost, toxicity and labor intensiveness present major challenges for DH line production. In addition, relatively few seeds are produced on each haploid and they therefore need to be propagated for another generation before they can be used in breeding applications. Also, as many of the induced haploids have a certain spontaneous doubling ability both in male and female, artificial chromosome duplication is not necessary for DH line production from germplasm with high proportion of fertile haploids (Kleiber et al. 2012).

Several researchers have reported the partial spontaneous duplication of haploids (Zabirova et al. 1993; Chalyk 1994; Geiger et al. 2006; Geiger and Schönleben 2011). They found variations in partial fertility recovery rates in different materials. Regarding the mechanism of spontaneous duplication of haploid plants, Testillano et al. (2004) studied chromosome doubling in early microspore embryogenesis in vitro and determined that fusion of nuclei likely leads to chromosome doubling. Moreover, spontaneous haploid doubling is always considered independently with haploid induction when taking into account double-fertilization mechanisms. Several mutants were found in efforts to describe the exact double fertilization process, like *feronia* (Huck et al. 2003), *sirene* (Rotman et al. 2003), *ig1* (*indeterminate gametophyte1*) (Evans 2007) and *cdka* (Nowack et al. 2006), and haploid inducers can also be considered as original mutant lines to help in the analysis of double-fertilization mechanisms (Li et al. 2009). The relationship between double-fertilization and spontaneous doubling is unclear.

In exception to this, the frequency of spontaneous chromosome doubling in some elite cultivars may be high enough to skip the doubling step and use the directly-obtained DHs (Kasha and Kao 1970; Hoekstra et al. 1993). In earlier study, researchers found that some haploids could spontaneously double in early development (Chalyk 1994). However, this phenomenon was not systematically studied and people did not pay much attention to its value in breeding program for a long time. In this study, these early doubled haploids were named as 'EH' and could be obtained in one step for use in maize breeding as pure lines. The objectives of this study were: (1) to report the phenomenon of early diploidization during in vivo haploid induction in maize; (2) to examine the homozygosity of the EH lines; (3) to provide insight into the biological mechanisms of spontaneous haploid doubling.

Materials and methods

Haploid induction

CAUHOI (Liu and Song 2000) was used as the haploid inducer line. The maize single-cross hybrid Zhengdan958, 55 families of its $F_{2:3}$ population and the parental lines Zheng58 and Chang7-2 were used as the female parents. Zhengdan958 was developed by the Henan Academy of Agriculture Science. The crosses were made manually at the Shangzhuang Experimental Station in Beijing in July 2010. The harvested putative haploids were screened by the marker gene *R1-nj* (Nanda and Chase 1966) and planted in Beijing in summer of 2011. All male-fertile haploid plants and haploid plants with tassel sectors were selfed.

Plant classification and morphological observation

The inducer line CAUHOI is homozygous for the dominant marker gene *plant purple coloration gene* (Liu and Song 2000), which leads to purple stalks and

leaves. Normally, the F_1 plants are vigorous because of heterosis and have purple stalks and leaves, whereas the haploid plants are shorter, with slender weak stems and narrower leaves, and grow slowly. However, a new kind of plant was observed before the flowering stage, showing green stalks and leaves but no heterosis, which was obviously not from contamination during the haploid induction process. We supposed these plants were doubled haploids that were doubled at an early stage. Hence, we named them early doubled haploids (EH). To study this new kind of plant, the plants from the putative haploids were classified into three groups: (1) haploid plants, which were short with green stalks and upright green leaves; (2) hybrid plants, which were strong with purple stalks and leaves; (3) EH plants, which had green stalks and leaves but were much stronger than haploid plants. During the flowering stage, all plants with completely fertile or partially fertile tassels were selfed. Morphological traits, such as plant height, ear height, tassel length and tassel branch number were measured at flowering time. Ear length, ear width and the number of selfed kernels were measured after harvest. To distinguish between EH and other DH lines, the DH lines generated from fertile haploid plants are named normal doubled haploids (DH) hereafter. The fertile EH plants were designated EH_0 and the harvested kernels were designated EH_1. The fertile haploid plants were designated DH_0 and the harvested kernels were designated DH_1.

Ploidy level determination using flow cytometry

To complement visual scoring of ploidy levels, three categories of plants at flowering time were examined with flag leaves by flow cytometry as described by Palomino et al. (2008) using a CAII flow cytometer (Partec GmbH, Münster, Germany): (i) 10 normal diploids and 10 completely male-sterile haploids as a control, (ii) 137 DH_0 plants, and (iii) 83 EH_0 plants. The DPAC software was used for data analysis. Nuclear ploidy was represented by C values, with 1C representing haploid, 2C representing diploid, and so on (Häntzschel and Weber 2010).

Simple sequence repeat (SSR) marker analysis

A total of 83 EH_0 plants and the parental lines of Zheng 58 and Chang 7-2 were chosen for SSR analysis. Maize genomic DNA was extracted from flag leaves at flowering time using the CTAB procedure (Saghai-Maroof et al. 1984). A total of 367 SSR primer sequences were screened for polymorphisms between Zheng58 and Chang7-2, Zheng58 and CAUHOI, and Chang7-2 and CAUHOI, respectively. Of these SSR markers, 104 SSR primer sequences showing clear polymorphisms in Zheng58 and Chang7-2, 107 in Zheng58 and CAUHOI, and 105 in Chang7-2 and CAUHOI (covering all 10 chromosomes) were used for SSR primer sequences analysis. All SSR primer sequences 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).

Evaluation of EH_1 lines for agronomic traits

Self-pollinated ears (EH_1) from EH_0 plants were manually harvested and ear traits were recorded on an individual plant basis. The ears were then grown ear-to-row in an observation nursery with up to 12 plants per row to visually evaluate plant uniformity. A set of 33 EH_1 lines from the Zhengdan958 haploids and the two parental lines were evaluated under normal conditions during the summer of 2012 in Beijing. A completely randomized block design was used, with three replications for each genotype. In each block, plants were sown in single-rows, 2.5-m long, with a density of 60,000 plants/ha. Unified management measures, such as irrigation, fertilization and weed cutting were applied during the whole growth period. Data were recorded on the following traits: plant height, ear height, tassel length, tassel branch number, ear width and ear length. Nine plants in the middle of each row were chosen for data collection.

Statistical analysis

For the agronomic traits (plant height, ear height, tassel length, tassel branch number, kernel number, ear width and ear length), analysis of variance (ANOVA) was performed by a generalized linear model (GLM) in SPSS (SPSS 11.5 for Windows) software. Means and variances of each EH and the controls were calculated. Mean comparison between EH and the checks was performed using Scheffe's test at 5 % level of significance. To confirm the homogeneity of

the EH_1 lines, the intra-variances (average of within-line variance) of the EH_1 lines and controls were obtained and evaluated using F-statistics. The parents, Zheng58 and Chang7-2, served as controls.

EH_0 in other inbred lines

To evaluate the EH frequency in other different materials, seven other elite inbred lines were also crossed with CAUHOI in the winter of 2011 in Hainan, including 4F1, B73, By815, Dan598, 8701, Qi319 and Xu178. The putative haploids of these materials (including Zheng58 and Chang7-2) were planted in Beijing and Gansu in the summer of 2012. Other haploids from Zhengdan958 were treated with colchicine and also planted in Beijing in 2012, the methods of treatment were refer to Häntzschel and Weber (2010). The EH_0 frequency was recorded for these materials and all fertile plants were selfed. EH_0 plants were also confirmed by flow cytometry and SSR markers. The proportion of EH_0 plants among haploid plants was called as EDR (early doubled rate) and the proportion of EH_0 plants among the seeds produced by the cross was called as EHI (early doubled haploid induction rate). A set of 5 EH_1 lines from each inbred line (Zheng58, Chang7-2, B73 and 8701) including the parents and their DH_1 lines were evaluated under normal conditions using a randomized complete block design (RCBD) with 3 replications in the winter of 2012 in Hainan (other five inbred lines were not be evaluated because of lack of DH lines). Each line was grown in single-rows, 2.5-m long, with a density of 60,000 plants/ha. Unified management measures were applied during the whole growth period. Data were recorded on the following traits: plant height, ear height, tassel length and tassel branch number. Nine plants in the middle of each row were chosen for data collection. The methods of statistical analysis were the same with way above.

Results

Morphology of EH_0

The performances of EH_0 plants were clearly different from either the normal haploids or hybrid plants. First, the EH_0 plants were more vigorous than normal haploids. The average values for plant height and ear height in EH_0 plants were 188 and 77 cm, which were significantly higher than the haploids (Fig. 1a; Table 1). Second, no heterosis was detected in the EH_0 plants but it was obvious in the hybrid plants, which had averages of 247 cm for plant height and 144 cm for ear height (Fig. 1a). In addition, hybrid plants had purple leaves and stalks as they were induced by CAUHOI with the *ABP1R* marker genes (*A1*: *anthocyanin gene*; *B*: *plant color strengthening gene*; *P1*: *plant purple coloration gene*; *R*: *plant aleurone color gene*) (Chase 1969; Liu and Song 2000), but no purple EH_0 plants were detected (Fig. 1a). Moreover, most DH_0 plants displayed only low levels of female and male fertility whereas all the EH_0 plants showed normal fertility. The means of ear width and length in EH_0 ears from Zhengdan958 were about 2–3 times greater than in DH_0 ears (Fig. 1b; Table 1). The seed setting was much better in EH_0 ears than in DH_0 ears. The EH_0 ear had averagely more than 100 kernels, whereas DH_0 ear had averagely less than 10 kernels (Fig. 1b; Table 1; Supplementary Table 1).

Ploidy level of EH_0 plants

Haploid plants had a significant peak at the C position (Fig. 2a), while normal diploid plants of the inbred line Zheng58 had a significant peak at the 2C position (Fig. 2b). Among the 83 EH_0 plants, all analyzed plants had a significant peak at the 2C position similar to that of normal diploids (Fig. 2c); however, there were three special EH_0 plants in which few haploid cells (1C) were detected (Fig. 2d), of which two from Zhengdan958 and one from $F_{2:3}$ family. Among the 137 DH_0 plants, 66 plants were detected with large numbers of haploid cells and a few diploid cells (Fig. 2e), 17 plants were detected with equal numbers of haploid and diploid cells (Fig. 2f), and the rest had only haploid cells (Table 2). The EH_0 plants were either in a primary diploid state (Fig. 2c) or a partial doubled state (Fig. 2d). Most of the normal spontaneously doubled haploid were in a haploid state (Fig. 2a) or primary haploid state (Fig. 2e), while a few were in a partial doubled state (Fig. 2f).

SSR marker analysis

No segments from CAUHOI were detected among the 83 EH_0 plants. The 7 EH_0 plants from the Zheng58 had

Fig. 1 Morphology of the progenies from Zhengdan958 × CAUHOI. **a** Three types of F_1 plants: *a1* hybrid plant with purple leaves and stem, *a2* early doubled haploid (EH) plant with green leaves and stalk, *a3* haploid plant, which was smaller than the diploid. **b** Selfed ears of fertile plants: *b1* selfed ears from EH plants, *b2* selfed ears from normal fertile haploid plants

Table 1 Means of normal spontaneously doubled haploids (DH_0) and early spontaneous doubled haploids (EH_0) for various morphological traits

	Total	Plant height/cm Mean	Ear height/cm Mean	Kernel number Mean	Ear width/cm Mean	Ear length/cm Mean
DH_0	45	111.45 ± 21.22a	45.89 ± 19.18a	7 ± 1.17a	1.45 ± 0.27a	6.57 ± 1.69a
EH_0	33	188.06 ± 24.4b	77.89 ± 15.43b	144 ± 21.33bc	3.78 ± 0.26b	14.09 ± 1.49b

the same genotype as Zheng58, and 17 EH_0 plants from Chang7-2 also had the same genotype as Chang7-2. From all loci characterized in the 33 EH_0s from Zhengdan958, 47.40 % of the alleles were Zheng58-type while 52.60 % were Chang7-2-type, producing the expected 1:1 ratio for the Zheng58 and Chang7-2 alleles. The alleles of the rest of EH_0 plants from Zhengdan958 $F_{2:3}$ were also from either parent. SSR analysis revealed 100 % homozygosis of all the EH_0 plants (Table 3; Supplementary Table 2).

Agronomic performance of the parents and variations within EH_1 lines

The two parents, Zheng58 and Chang7-2, greatly varied and differed from each other in the agronomic characteristics being investigated (Table 4). Zheng58 differs substantially from Chang7-2 in five of the investigated traits such as: plant height, ear height, tassel branch number, ear length and ear width. Highest variation was observed in plant height followed by ear height in the two parents. Chang7-2 was taller with the plant height of 188.88 cm and the ear height of 83.46 cm as compared to Zheng58 with 160.00 and 59.94 cm. The value of EH_1 lines and value of mid-parents differed in plant eight, ear height, tassel branch number and ear length, but not statistically significant. The EH_1 lines showed a tendency of slightly taller mean height, a little longer ear and less tassel branch number.

No off-type plants were observed within EH_1 lines. The homogeneity of within-line populations was further examined by comparing the intra-variance of each EH_1 line against the pooled variance of the parents. The F values, determined by the ratio of the intra-variance of the EH_1 lines versus the pooled variance of the controls, did not reveal any significant differences in any of the traits tested at a 5 % level of significance (Table 4). These indicated that each EH line behaved in the same manner and showed the same potentialities as the parents Chang 7-2 and zheng58, which were developed through traditional breeding and evolved through time by ordinary selfing. Theoretically, it is believed that EH is genetically stable.

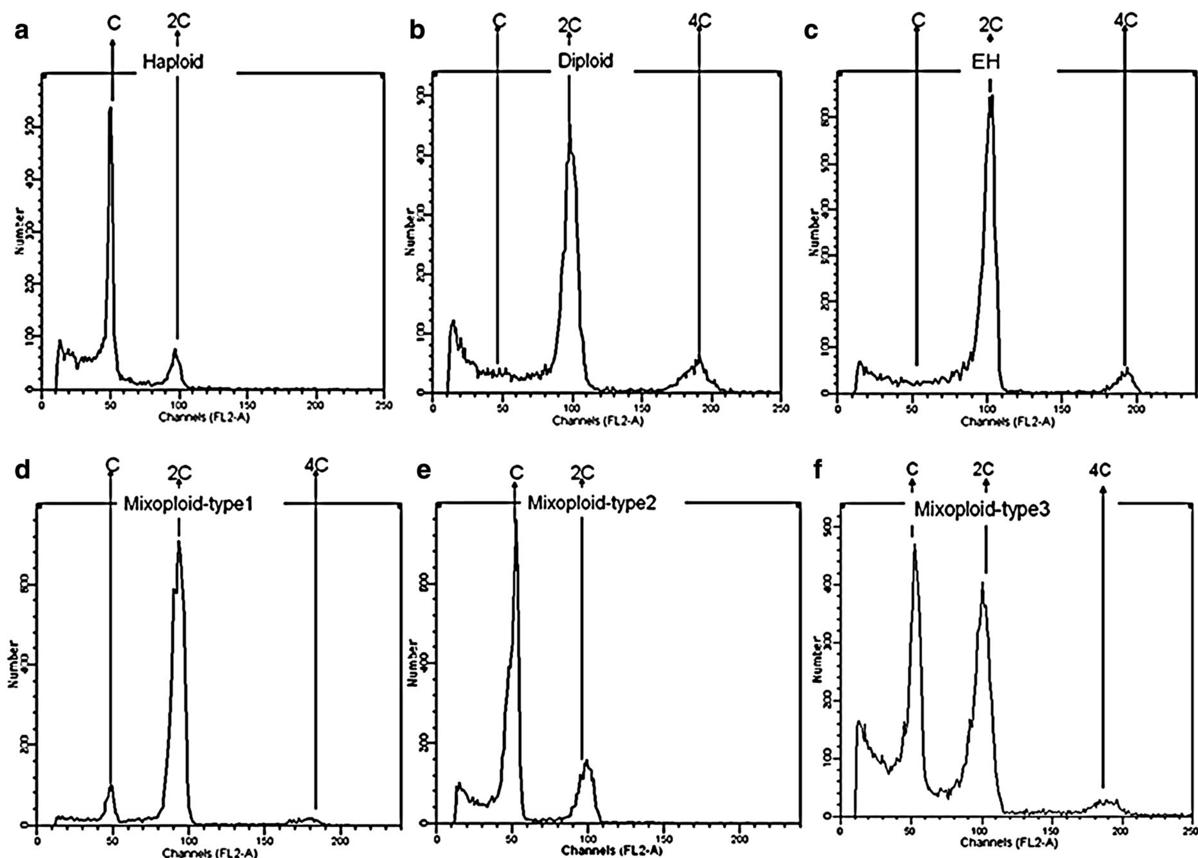

Fig. 2 Determination of ploidy level using flow cytometry according to the position of peaks representing the size of nuclei, as determined for flag leaves at the flowering stage. **a** Haploids. **b** Normal diploid plants of the inbred line Zheng58. **c** Early doubled haploids (EH_0). **d** EH_M, Mixoploid-type 1 was mixoploid in diploid EH plants, with few haploid cells and many diploid cells. **e** Mixoploid-type 2 was chimeric and contained large numbers of haploid cells and few diploid cells. **f** Mixoploid-type 3 had equal amounts of haploid and diploid cells. *Note* the position of the first peak on the left determines the ploidy. C, haploid cells in G1/G0 phase; 2C, diploid cells in G1/G0 phase and haploid cells in G2/mitosis; 4C, diploid cells in G2/mitosis

Table 2 Numbers of EH_0 and DH_0 plants induced from Zhengdan958, the Zhengdan958 $F_{2:3}$ population and the parents with CAUHOI as the inducer line among different ploidy level types, as determined by flow cytometry

Material/type	Total	Diploid-type	Haploid-type	Mixoploid-type 1[a]	Mixoploid-type 2[b]	Mixoploid-type 3[c]
EH_0	83	80	0	3	0	0
DH_0	137	0	44	0	66	17

[a] Mixoploid-type 1 was a chimera with few haploid cells and large numbers of diploid cells

[b] Mixoploid-type 2 was a chimera with large numbers of haploid cells and few diploid cells

[c] Mixoploid-type 3 was a chimera with equal numbers of haploid and diploid cells

Frequency of early doubled haploids

On the one hand, as for the proportion of EH_0 plants among haploid plants (EDR), the average frequencies of confirmed EH_0 plants from Zheng58 haploids, Chang7-2 haploids, Zhengdan958 haploids and the Zhengdan958 $F_{2:3}$ families were 2.24, 3.76, 3.52 and 3.21 %, respectively (Table 5). Higher frequency of EH_0 plants was detected from Chang7-2 than Zheng58. The rate of EH_0 occurrence in the Zhengdan958 was

Table 3 Frequency of the alleles of parents, heterozygous and inducer in 33 EH_0s generated from the F_1 of Zhengdan958

Material	Heterozygous[a]	Zheng58[b]	Chang7-2[c]	Inducer[d]
$EH_0$1	0	45.63	54.37	0
$EH_0$2	0	62.14	37.86	0
$EH_0$3	0	52.94	47.06	0
$EH_0$4	0	42.00	58.00	0
$EH_0$5	0	53.47	46.53	0
$EH_0$6	0	50.00	50.00	0
$EH_0$7	0	48.98	51.02	0
$EH_0$8	0	51.92	48.08	0
$EH_0$9	0	62.00	38.00	0
$EH_0$10	0	53.85	46.15	0
$EH_0$11	0	47.57	52.43	0
$EH_0$12	0	60.58	39.42	0
$EH_0$13	0	65.05	34.95	0
$EH_0$14	0	46.94	53.06	0
$EH_0$15	0	48.54	51.46	0
$EH_0$16	0	61.39	38.61	0
$EH_0$17	0	35.58	64.42	0
$EH_0$18	0	64.71	35.29	0
$EH_0$19	0	42.31	57.69	0
$EH_0$20	0	54.81	45.19	0
$EH_0$21	0	53.85	46.15	0
$EH_0$22	0	53.40	46.60	0
$EH_0$23	0	51.96	48.04	0
$EH_0$24	0	54.81	45.19	0
$EH_0$25	0	44.12	55.88	0
$EH_0$26	0	56.73	43.27	0
$EH_0$27	0	55.45	44.55	0
$EH_0$28	0	50.60	49.40	0
$EH_0$29	0	65.38	34.62	0
$EH_0$30	0	53.06	46.94	0
$EH_0$31	0	35.58	64.42	0
$EH_0$32	0	47.12	52.88	0
$EH_0$33	0	63.46	36.54	0

[a] Frequency of alleles Heterozygous-type in EH_0 plants through analysis of SSR markers

[b] Frequency of alleles Zheng58-type, one of the parents, in EH_0 plants through analysis of SSR markers

[c] Frequency of alleles Chang7-2-type, one of the parents, in EH_0 plants through analysis of SSR markers

[d] Frequency of alleles Inducer-type in EH_0 plants through analysis of SSR markers

between those of the Zheng58 and Chang7-2. The average frequency of EH_0 in these different genetic materials ranged from 1 to 3.5 % (Table 5). The frequency of EH in this artificially treated population was similar (Table 5) to the frequency in spontaneous doubled haploids. On the other hand, however, for the proportion among all of the induced seeds (EHI), the rate was very low. All of the materials' EHI were no more than 0.1 % (Table 5) because of the low HIR of CAUHOI.

Discussion

The production of DH lines makes it easy to carry out genetic studies and significantly shortens the breeding time (Seitz 2005). DH technology has become the routine breeding strategy in many commercial maize breeding programs (Geiger and Gordillo 2009; Prigge and Melchinger 2011). Many works contributed to the haploid induction (Li et al. 2009; Wu et al. 2014; Zhao et al. 2013) but only a few works cared about the haploid doubling. Normally at least two seasons are necessary in the current DH production scheme. One season is haploid induction from manual crossing or in isolation and afterwards haploid kernel identification depending on special marker such as *R1-nj* (Chase 1969), KOC (Chen and Song 2003; Melchinger et al. 2013), and so on. The second season is haploid doubling, spontaneously or some chemical agents being used. Theoretically DH_1 lines can be used for further observation as well as testcross production. However, DH_1 lines usually cannot be directly used in further tests due to the low number of seeds on selfed ears, hence normally one additional season is required. Recent studies have shown that the mean number of intact seeds on selfed ears (IS) after artificial treatment was only 3.82 (Kleiber et al. 2012). On the other hand, chemical doubling is time consuming and expensive in many cases. The alternative economic and environmental friendly method of DH production which can produce enough seed kernels after doubling is very important in DH breeding scheme.

Chalyk (1994) earlier found some "unusual plants phenotypically resembling homozygous lines" and assumed that the plants resulted from spontaneous chromosome doubling in haploids. By way of explanation, he suggested that unusual plants occurred at "early ontogenetic stages". He listed three characteristics of the usual plants: a) they lack heterosis, b) the uniformity of their progeny does not differ from that of inbred lines, and c) they lack dominant marker genes

Table 4 Mean values for parents (Chang7-2 and Zheng58), mid-parent, and early doubled haploid (EH_1) lines from Zhengdan958; and F values for variances within EH_1 lines in comparison with the checks for agronomic traits for six traits (plant height, ear height, tassel length, tassel branch number, ear width and ear length)

Trait	Mean				F[a]	Significance of difference
	Chang7-2	Zheng58	Mid-parental value	EH lines		
Plant height/cm	188.88 ± 9.60	160.00 ± 7.95	174.44	185.48 ± 8.25[b]	0.05	NS
Ear height/cm	83.46 ± 8.46	59.94 ± 7.06	71.7	77.12 ± 6.90	0.19	NS
Tassel length/cm	24.62 ± 3.18	24.50 ± 3.25	24.56	23.63 ± 2.07	1.12	NS
Tassel branch number	22.31 ± 4.45	6.50 ± 1.43	14.41	10.16 ± 2.04	0.61	NS
Ear width/cm	4.14 ± 0.41	3.88 ± 0.19	4.01	3.91 ± 0.22	1.31	NS
Ear length/cm	10.06 ± 0.63	14.5 ± 0.13	12.28	14.16 ± 1.44	2.51	NS

NS not significant. Significant at $p < 0.05$

[a] Ratio of an average of within EH lines variance versus pooled variance of the checks

[b] An average of standard deviation for each EH_1 line

Table 5 The frequency of early doubled haploid (EH) plants determined from different materials

Material		Total seed	Haploid	EH	HIR %	EDR (%)[a]	EHI (%)[b]
Zhengdan958	Beijing	35,799	712	25	1.99	3.51	0.07
	Gansu	10,966	227	8	2.07	3.52	0.07
	A.T.	10,647	214	7	2.01	3.27	0.07
8701	Beijing	9,290	157	3	1.69	1.91	0.03
	Gansu	9,296	185	4	1.99	2.16	0.04
4F1	Beijing	7,410	186	2	2.51	1.08	0.03
	Gansu	6,022	162	2	2.69	1.23	0.03
Zheng58	Beijing	6,109	190	4	3.11	2.11	0.07
	Gansu	4,006	127	3	3.17	2.36	0.07
Chang7-2	Beijing	13,103	190	7	1.45	3.68	0.05
	Gansu	16,774	260	10	1.55	3.85	0.06
Xu178	Beijing	6,716	135	3	2.01	2.22	0.04
	Gansu	7,087	146	2	2.06	1.37	0.03
Qi319	Beijing	6,039	125	3	2.07	2.40	0.05
	Gansu	6,303	133	2	2.11	1.50	0.03
BY815	Beijing	9,685	215	3	2.22	1.40	0.03
	Gansu	10,324	223	3	2.16	1.35	0.03
B73	Beijing	11,232	310	4	2.76	1.29	0.04
	Gansu	7,189	202	3	2.81	1.49	0.04
Dan598	Beijing	4,871	189	2	3.88	1.06	0.04
	Gansu	3,504	137	2	3.91	1.46	0.06

A.T. artificial Treatment (colchicine)

[a] EDR = (EH plants/total haploid plants in the field) × 100 %

[b] EHI = (EH plants/total induced seeds) × 100 %

of the pollen parent (Chalyk 1994). Chalyk compared the progeny of these plants with five-selfing inbred lines and found that ear height and ear width of these plants were statistically significant different with inbred lines, so he believed that the inbred lines were quite favorable. And for a long time, no relatively

studies reported the similar phenomenon. In our study, not only cytological observation revealed these plants were diploid plants, but also morphological and molecular analysis of EH revealed that they were as homozygous as normal DH lines and their parents. It revealed homozygosity for 100 % of the total marker loci in the 83 EH_0 plants. The EH_1 lines from Zhengdan958 behaved in the same manner and showed the same potentialities as the parents of Zheng58 and Chang7-2 (Table 4). This was consistent with the results of Chalyk. The comparison between inbred lines and their EH lines showed that the mean values for most of agronomic traits were lower in EH_1 lines than in the parental lines (except B73 in tassel branch number and 8701 in ear height and tassel branch number) (Supplementary Table 3). This may be from the poorer seed vigor of EH lines or some residual heterozygote loci in inbred lines. However, the differences are not significant. The selfed ears of EH_0 could produce enough kernels for the next step such as observation and seed production in breeding program (Fig. 1b; Table 1 and Supplementary Fig. 1).Our study showed that there was no difference between EH_1 and DH_1 lines (Supplementary Table 3) in agronomic performance (Sugimoto and Arai 2002) for potential use but enough seed kernels of EH can be obtained in one step just after haploid induction. Hence, our research may represents a breakthrough in DH technology of maize research and breeding.

EH_0 plants were obviously different from DH_0 plants in the agronomic performances (Fig. 1). This was also reflected by the degree of diploidization according to flow cytometry e examination. All of these DH_0 plants showed incomplete diploidization of the haploid cells whereas the EH_0 plants showed complete diploidization. The doubling processes between EH and DH are most probably different. On the one hand, in this study, there was little association between the rate of normal spontaneous doubling (data not shown) and the rate of EH. For example, in the line 8,701 more than 80 % of haploids showed fertile pollen and less than 3 % (EDR) of plants were EH_0 but in Chang7-2 less than 30 % of haploids were fertile and more than 3 % (EDR) of plants were EH_0 (Table 5). On the other hand, the frequency of EH did not increase in conjunction with the increased doubling rate after treatment with a chemical reagent. The fertile haploids produced via chemical (colchicine) treatment were also found to be incomplete diploids and the frequency of EH in this artificially treated population was similar (Table 5) to the frequency in spontaneous doubled haploids. Hence, we speculate that the mechanism of EH formation may be different from the mechanism of normal spontaneous haploid doubling or that EH formation may happen at a different stage from normal spontaneous haploid doubling but occur by the same mechanism. The artificial treatment normally was done during seed germination or in the seedling stage. Even though, the diploidization from artificial treatment is not as complete as EH. This implicates that EH formation probably occurs before seed germination or during embryo development after induction pollination.

There are two possible ways that EH plants could arise. One is that maternal materials produce 2n female gametes by abnormal meiosis before the induction process and these female gametes develop into diploids by parthenogenesis. The other possibility is that EH can arise from normal n gametes or haploid zygotes produced during the induction by the cross between maternal materials and inducer lines, and in subsequent seed development, the genomes of the embryos were doubled and developed into diploids through abnormal mitosis processes (Mittwoch 1978; Segui-Simarro and Nuez 2008). Higher plants spontaneously produce 2n gametes at a low frequency (no more than 0.6 %) (McCoy et al. 1982; Lux et al. 1990; Bohanec et al. 1995; Miyoshi and Asakura 1996; Ficcadenti et al. 1999); Bauman (1961) found that 2n egg cells were produced by abnormal meiosis in a number of maize hybrids at a frequency of 0.058 % to 0.523 % in the crossing of a single cross hybrid with a tetraploid. Many factors can lead to abnormal meiosis that results in the formation of 2n gametes, such as meiotic nuclear restitution, second division spindle healing and cytokinesis abnormalities. It seems that this possibility could not be excluded as EHI (the proportion of EH plants among all induced seeds) may be close to frequency of 2n gametes frequency reported before. However, all of the planted seeds had obvious *R1-nj* markers on the top of the embryo and this means that EH could not originate from pathogenesis. And in this study, three mixoploids (EH_M) were detected among the EH_0 plants, in which less than 10 % of the cells were haploid (Fig. 2d). The occurrence of these kinds of mixoploids suggests that the egg or zygote that gave rise to these plants may have been haploid, not diploid, as a few haploid cells

were not completely doubled. Therefore, EHs may have been formed through the second process.

Normally, spontaneous duplication of a haploid genome is thought to occur mainly through three mechanisms: endoreduplication, nuclear fusion and endomitosis (Meyer 1925; Hu and Kasha 1999; Testillano et al. 2004; Shim et al. 2006; Segui-Simarro and Nuez 2008). If all of these results indicate that EH formation differs from the random formation of 2n gametes and represents a special doubling process originating from haploid gametes or zygotes, then the interesting question is whether the haploid comes from a fertilized zygote or an unfertilized maternal gamete. Two mechanisms leading to the formation of maternal haploids have been proposed: (1) one of the two sperm cells that are present during double fertilization is not able to fuse with the egg cell but can trigger haploid embryogenesis. The second sperm cell fuses with the central cell, leading to the formation of a regular triploid endosperm. (2) One of the two sperm cells provided by the inducer is defective but is still able to fuse with the egg cell. During subsequent cell division, the inducer chromosomes degenerate and are eliminated stepwise from the primordial endosperm. The second cell fuses with the central cell, as described in the first hypothesis (Geiger and Gordillo 2009; Li et al. 2009). From our speculation, all spontaneous doubling mechanism might lead to the production of the early doubled haploid. As described above, chromosome elimination could make donor cell chromosomes 'unstable', resulting in 'internal division' of the chromosomes themselves combined with a failure in the assembly of the mitotic spindle. This process would happen exactly after normal duplication of the chromatids during S-phase and be induced by the male parent. However, endoreduplication and nuclear fusion need not be exclusive as induction could cause inhibition of mitosis (M-phase) or chromosomes to pass in and out of the cell wall to make it intact because of elimination.

The last but important thing we should mention was that the rate of EH occurrence did not very high: early doubled rate (EDR) was less than 4 % in our detected materials and this rate was consistent with the results of Chalyk in 1994 in observing the unusual plants, which was not high enough to reach the reported average spontaneous haploid doubling rate 10 % (Chase 1952; Chalyk 1994; Barnabas et al. 1999), not to mention the efficiency of colchicine treatment; moreover, the other index which Chalyk did not mention was the early doubled haploid induction rate (EHI). Compared to the average haploid induction rate (about 2 %) of the materials in our study, the EHI was even less. In this situation, EH should be used in plant breeding after further effective studies or technologies to explore to increase the rate. Therefore, taking into account the possible way that the EH plants could arise, we considered that special inducer that could produce enough early doubled haploids would be a new area in further DH breeding. Indeed, the study of EH provides an excellent opportunity to elucidate the mechanisms underlying haploid inducing and doubling. Further work is also needed to determine the exact process of spontaneous haploid doubling. Such work will provide new information about the factors that affect double fertilization in flowering plants.

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Conflict of interest The authors declare that they have no conflict of interest.

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