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

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ABSTRACT

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

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1. Introduction

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

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

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

Researchers have focused on two possible mechanisms: parthenogenesis and chromosome elimination. Firstly, parthenogenesis was caused by the irregularities of microsporogenesis and fertilization [20–25]. All of these findings indicate that various irregularities appearing between microsporogenesis and fertilization may prevent double fertilization and stimulate division of the egg cell without fertilization. As a result of this process, a haploid embryo can be formed from an unfertilized egg cell. Secondly, a set of chromosome that is randomly eliminated after fertilization might be a major mechanism underlying *in vivo* haploid-induction in maize. Wedzony et al. [17] observed that 10% of the resulting

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

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

2. Materials and methods

2.1. Plant materials and pollination

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

2.2. Sampling methods and cytology

2.2.1. Sample, fixation and isolation

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

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

2.2.2. Microscopic examination

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

2.2.3. Fertility investigation

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

2.3. SSR analysis

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

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

3. Results

3.1. Identification of fertility and morphology

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

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

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

In this study, the seed setting rate from self-fertilization or crosses among HZ514, HZI1, NA and 248 were significantly different. The seed setting rate of HZ514 self-fertilization, HZ514 × NA, HZ514 × 248 and HZI1 × HZ514 were normal. However, when HZI1 was used as the male parent for either cross or self-cross, the seed setting rate was very poor and some kernels were abnormal in morphology (Fig. 1).

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