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# Haploid induction and its application in maize breeding

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**Abstract** Maize is a heterosis-utilizing crop species, and the application of maize hybrids has significantly improved total maize yields worldwide. Breeding pure lines is the most important part of heterosis utilization. The double haploid (DH) breeding technology is the approach rising recently in breeding pure lines; compared to the conventional recurrent-selfing method, it can significantly accelerate the crop breeding process. Similar to molecular breeding and transgenic techniques, maize DH breeding has been playing an increasingly important role in commercial breeding and is becoming the core technique in modern maize breeding. In this review, we summarize recent progress in maize DH breeding and put forth our opinions on the future development of double haploid techniques in modern maize breeding.

**Keywords** Maize breeding · Haploid · Inducer lines · DH lines

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Pure line production is an important component of maize breeding programs as parents of hybrid lines (Chaikam et al. 2019). Trait separation would occur in the F1 hybrid generation using heterozygous lines as parents. On the contrary, the plant traits of F1 populations would show uniform when using the pure lines as parents, which would achieve the goal of the stable yield. Currently, two approaches are commonly adopted in breeding pure lines: one is the conventional recurrent-selfing method, which usually requires 6 to 8 generations to produce a homozygous line, and the other is double haploid breeding, which can rapidly generate pure lines within only two generations via the DH technique (Geiger 2009). Haploids refer to individual plants with a gametic chromosome number in their cells. Since haploids have only one set of chromosomes, the effects of both recessive and dominant genes can be seen in their generation; thus, it is easier to select individuals with excellent traits and eliminate those with poor traits in a timely manner (Forster et al. 2007; Geiger and Gordillo 2009). The DH technique can also be applied for generating mapping populations and chromosome substitution lines and for reverse breeding and apomixis engineering (Wijnker et al. 2014). This

review focuses on the haploid generation, induction mechanism of stock 6, haploid identification, haploid doubling, and emerging applications in maize breeding process.

## Generation of haploids

Five approaches are usually adopted to generate plant haploids: tissue culture (Wang et al. 2000; Chen et al. 2016), natural generation (Goodsell 1961; Chase 1963), CENH3-mediated haploid induction (Kelliher et al. 2016), induction by haploid inducer lines (Coe 1959; Evans 2007), and interspecies hybridization (Kasha and Kao 1970; Barclay 1975; Laurie and Bennett 1988). Four of them are utilized to produce maize haploids, the first of which involves obtaining haploids by tissue culture from megasporocytes and microsporocytes of maize (Barloy 1989; Petolino et al. 1992). Ao et al. culture the maize unpollinated ovaries on MS and N6 medium and obtain nearly 2% haploid plants (Ao et al. 1982). However, this method is rarely used due to its high dependence on the parental genotype, high possibility of mutations in the culture process, and prolonged duration (Chaikam 2012).

Second, *indeterminate gametophyte* (*ig*) mutants have been used to generate haploids. The *ig* gene interferes with the function of the cytoskeleton in mitosis during the multinucleated cell stage after meiosis of the embryo sac, resulting in disordered cell division (Kermicle 1969; Lin 1981; Enaleeva et al. 1995). A certain proportion of male or female haploids are produced by crossing between the *ig* mutant and common inbred lines. However, in some backgrounds, the homozygous *ig* mutant is sterile, which makes using the *ig* gene highly inconvenient (Evans 2007).

The third approach is CENH3-mediated haploid induction. CENH3 is essential for kinetochore and spindle attachment during mitosis and meiosis (Kuppu et al. 2020). When a line with small or defective centromeres is crossed with a line with large or normal centromeres, the smaller or defective centromeres will be selectively degraded, resulting in a loss of chromosomes from the small-centromere parent (Bennett et al. 1981; Rieralizarazu et al. 1996; Zhang and Dawe 2012; Wang and Dawe 2018). Transgenic *Arabidopsis* (*Arabidopsis thaliana*) plants containing modified CENH3 can induce haploids when hybridized with wild-type plants (Ravi and Chan 2010; Ravi et al. 2011; Ravi et al. 2014; Britt and Kuppu 2016). Based on this discovery in *Arabidopsis*, Kelliher et al. carried out that nearly 0.86% of the seeds found in the hybrids of *cenh3* mutant materials were haploid. This study demonstrated that CENH3 can be used to generate parthenogenetic maize haploids. Furthermore, because all of the plants have homologs of CENH3, this method may be extended to other crop species (Kelliher et al. 2016; Kuppu et al. 2020).

The fourth approach involves induction by inducer lines. To date, nearly all in vivo maize haploid inducer lines have been derived from the same ancestor line, stock 6. This line can produce female haploids with a 1–2% induction rate (Coe 1959). The origin of stock 6 is a Mexican farm species with white endosperm, a purple aleurone layer, and a hard kernel type. As stock 6 has a lower induction rate and many defects in terms of agronomic traits, many improved inducer lines have been developed, such as WS14 (Lashermes and Beckert 1988), KEMS (Sarkar et al. 1994), MHI (Chalyk 1999), RWS (Röber et al. 2005), and CAU5 (Chen 2012). Since stock 6-driven haploid inducer lines have been widely used in maize breeding, this review mainly focuses on studies about maternal haploid inducer lines derived from stock 6.

## Mechanisms underlying maternal haploid induction by inducer lines derived from stock 6

For the haploid inducer lines derived from stock 6 and its derivatives, two hypotheses are currently used to explain their induction mechanisms: the single-fertilization hypothesis and the chromosome elimination hypothesis.

The hypothesis of single fertilization was put forth first (Sprague 1929; Sprague 1932). In this hypothesis, one sperm cell from the inducer gamete fails to fuse with the egg cell but triggers haploid embryogenesis. Only a few studies have been conducted to support this hypothesis. Bylich et al. found that 6.32% of the pollen in the inducer line ZMS showed differences in sizes of two sperm. They speculated that one sperm cell successfully fertilizes either the endosperm or embryo, whereas the other does not because it is defective, which led to single fertilization (Bylich and Chalyk 1996). Chalyk et al. found that nearly 15% of the microsporocytes in MHI inducer lines were aneuploid but that only 1% were aneuploid in inbred lines; thus, the authors speculated

that the haploid phenomenon might be triggered by aneuploidy (Chalyk et al. 2003). Moreover, other studies have revealed that haploid induction might be correlated with heterofertilization. Haploid inducer lines can produce more heterofertilization kernels when they are used as male parents (Rotarenco and Eder 2003). Tian et al. found that in abnormal ovaries of immature embryos, no embryo structure was produced despite an obvious endosperm structure, which suggested that single-fertilization events occurred (Chen et al. 2016; Tian et al. 2018).

According to the chromosome elimination hypothesis, the two sperm cells from a single pollen of a haploid inducer line fuse with the egg cell and polar nuclei separately, but the chromosomes from the inducer line are eliminated during subsequent zygotic divisions. Several lines of evidence support this hypothesis, including observations of micronuclei in some of the ovules fertilized by inducer pollen and the presence of a rare inducer DNA fragment detected in certain haploid seeds (Wedzony et al. 2002; Fischer 2004; Li et al. 2009; Qiu et al. 2014). Interestingly, a small number of haploids carrying the B chromosome were found when the inducer lines contained the B chromosome and were used as male parents (Zhao et al. 2013). In addition, Li et al. found that chromosome fragmentation is related to haploid induction and that the process of fragmentation is gradual, so they proposed a model of the induction mechanism in maize according to the severity of chromosome fragmentation within two sperm (Li et al. 2017). The type I model involves normal or slightly fragmented sperm fusing to normal eggs to form diploids. In type II, haploids can be produced only when the sperm fertilizes the egg and has undergone chromosome fragmentation, whereas abortive kernels are produced if this sperm fertilizes the central cell. Moreover, in type III, severe chromosome fragmentation occurs in both sperm, ultimately causing abortion of the entire kernel (Fig. 1). Although more research has supported the chromosome elimination hypothesis, this hypothesis does not contradict the single-fertilization hypothesis. Chromosome elimination and single fertilization might occur simultaneously in the haploid induction process.

Studies have shown that the induction ability of stock 6 is genetically controlled by 8 QTLs (Lasherme and Beckert 1988), among which *qhir1* and *qhir8* accounted for 66% and 20% of genetic variation, respectively. The inducer line UH400, with a high induction rate, and a non-inducer line, 1680, were then used to construct a mapping population to locate the gene within *qhir1*, and the mapping region of *qhir1* was ultimately narrowed to a 243-kb region (Dong et al. 2013). Recently, the stock 6-derived gene affording haploid induction was cloned by three independent groups, and gene was named

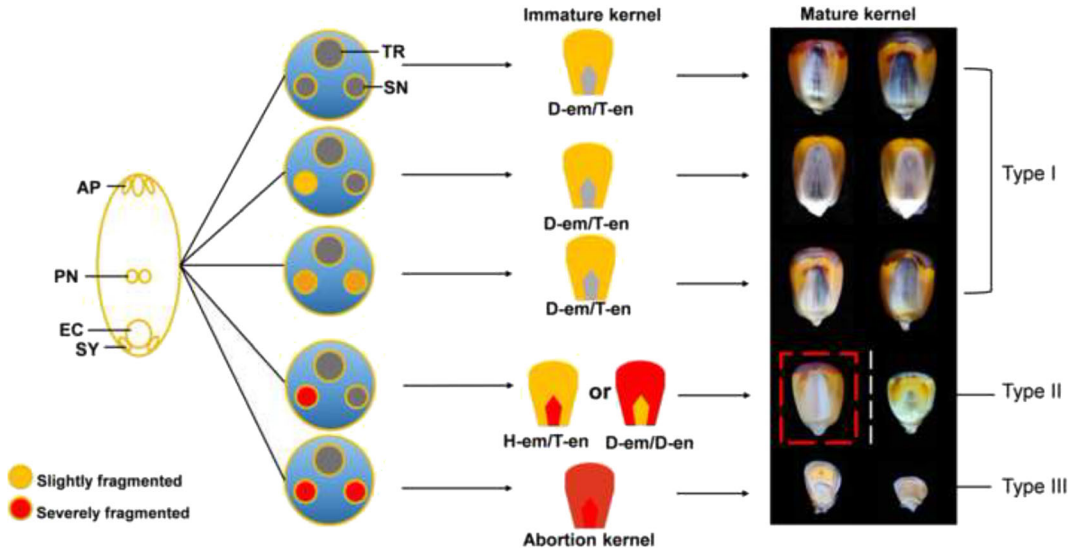


**Fig. 1** Model of the induction mechanism of maize. Type I: The normal or slightly fragmented sperm fused with normal eggs form diploids; type II: One of the sperm that has undergone chromosome fragmentation and has fertilized an egg may form haploids and abortive kernels; type III: Severe chromosome fragmentation occurs in both sperm and abortion kernels that have formed. AP, antipodal cell; PN, polar nucleus; EC, egg cell; SY, synergid cell; TR, trophic nucleus; SN, sperm nucleus; D-em, diploid embryo; H-em, haploid embryo; D-en, diploid endosperm; T-en, triploid endosperm

*MATRILINEAL* (*MTL*), *ZmPHOSPHOLIPASE A1* (*ZmPLA1*), and *NOT LIKE DAD* (*NLD*), respectively. A 4-bp insertion in the last exon causes a frameshift mutation and the early truncation of the encoded protein (Kelliher et al. 2017; Liu et al. 2017; Gilles et al. 2017). Knockout and RNAi of the *ZmPLA1* gene by the CRISPR/Cas9 technique could induce nearly 2% haploids, which verified that *GRMZM2G471240* was the target gene (Kelliher et al. 2017; Liu et al. 2017; Gilles et al. 2017).

Liu et al. mapped the second major QTL, *qhir8*, to a 789-kb region in 9.01 bin (Liu et al. 2015). The final mapping region was located within the protein-coding sequence of the gene *GRMZM2G465053*, which encodes a DUF679 domain membrane protein and was named *ZmDMP* (Zhong et al. 2019). A single-nucleotide change in *ZmDMP* leads to a 2- to 3-fold increase in the HIR. Knockout of *ZmDMP* triggered haploid induction, with an HIR of 0.1 to 0.3%, and there was a greater ability to increase the HIR by 5- to 6-fold in the presence of *mtl/pla/nld* (Zhong et al. 2019). The cloning of these two induction genes provided important information for studying the molecular mechanism of haploid induction and improving the DH breeding efficiency in maize.

## Identification of maize haploids

An in vivo haploid induction system produces diploid and haploid kernels simultaneously, and it is necessary to identify haploids from diploids by the use of unique markers. Currently, haploid identification is mainly carried out during the seed and seedling stages.

### Haploid seed identification

The $R_1$-nj marker is the most commonly used color marker in haploid identification at the seed stage. In 1966, Sarkar and Coe introduced the $R_1$-nj color marker into inducer lines and then used purple markers expressed in the aleurone layer and scutellum to identify haploids, where haploid kernels have a colorless scutellum and a pigmented aleurone (Sarkar and Coe. 1966). However, the genetic background of the maternal parents interfered with the accuracy of identification. In particular, flint and tropical germplasms often contain the gene *C1-I*, which inhibits the pigmentation of $R_1$-nj (Röber et al. 2005). As such, the oil content (OC) classification system has been proposed as an alternative way to distinguish haploid and diploid kernels without interference from genetic backgrounds (Chen and Song 2003). Chen and Song showed that the OC of crossed diploid maize kernels was 30% greater than that of haploid kernels from ears pollinated by the high-oil-inducer line CAUHOI (Chen and Song 2003). This haploid identification method was more than 90% accurate, even among various genetic backgrounds (Dong et al. 2014). Furthermore, the use of inducer lines containing fluorescence markers may constitute another method for seed identification (Zhao et al. 2013; Dong et al. 2018).

### Haploid seedling identification

The methods of haploid identification at the seedling stage include morphological identification, chromosome counting, and the use of specific markers. The morphological identification method mainly focuses on the characteristics of haploid plants with a short stature, small leaf angles and widths, and poor pollen fertility (Chase 1964; Chase 1969; Wu et al. 2016; Liu et al. 2017). However, morphological identification has rarely been used for the large-scale production of DH lines due to inefficiency. It requires planting and evaluating large numbers of induced seed, which is a time-consuming process (Belicuas et al. 2007; Chaikam et al. 2019). In the lab, the ploidy level can be evaluated via flow cytometry (Bohanec 2003), and chromosome number can be determined by the root tip squashing method (Zhao et al. 2013). In addition, parents with specific markers, such as Basta-resistant inducer lines, can also be used for haploid identification (Geiger 2009).

### Doubling of haploids

In maize breeding, only DH lines instead of haploids are used; haploids are usually sterile (Chaikam and Mahuku 2012), as meiosis cannot occur and leads to abnormal gamete formation (Chalyk 1994). Therefore, the production of fertile pollen from haploids is considered a critical factor for DH line production (Kleiber et al. 2012; Wu et al. 2016; Ren et al. 2017). Generally, male fertility can be restored by spontaneous chromosomal doubling or artificial chromosomal doubling using chemical reagents.

### Spontaneous chromosomal doubling

A few haploid plants can produce fertile pollen under natural conditions (Chase 1949). In most maize germplasms, the rate of spontaneous chromosome doubling is lower than 1% (Kleiber et al. 2012). The environment can affect the rate of haploid doubling. For example, the doubling rate in Hainan (N. 18° 15′, E. 109° 30′) was higher than that in Heilongjiang (N. 43° 26′, E. 121° 11′) (Cai et al. 2012). Recent studies have shown that the spontaneous doubling rate is also influenced by genetic variation and high heritability, so it is possible to improve the rate by selection (Kleiber et al. 2012; Wu et al. 2016; Ren et al. 2017; Ma et al. 2018; Jiao et al. 2020). Seven loci controlling male fertility of haploids were detected by assessing a constructed mapping population (Wu et al. 2014), and one of the loci, *qhmf4*, was narrowed to within a 600-kb region (Ren et al. 2017). Mapping of the fertility-restorer genes will further improve the efficiency of spontaneous doubling.

### Artificial chromosomal doubling

Artificial chromosome doubling is carried out by treating haploid seeds, seedlings, or immature embryos with chemicals that have antimitotic activity, including colchicine, herbicides, and $N_2O$. Colchicine binds to β-tubulin and prevents the formation of tubulin dimers. This chemical also prevents the replication, separation, and polar shift of replicating chromosomes as well as cell division, resulting in the doubling of cell chromosome numbers (Chase 1952; Gayen and Sarkar 1996). However, colchicine is highly toxic and not only has a potential carcinogenic effect but also is harmful to the environment (Chaikam and Mahuku 2012; Melchinger et al. 2016). Fortunately, compared with the success rate of colchicine, some low-toxicity chemical reagents that induce similar rates of chromosome doubling have been identified, such as dimethyl sulfoxide (DMSO), methamidophos (APM), and propanolamine (Zhao and Gu 1984; Wan et al. 1991; Beaumont and Widholm 1993; Guo et al. 1997; Murovec and Bohanec 2012). In addition, another alternative reagent for colchicine is $N_2O$ (Kato 1997; Molenaar et al. 2018; Molenaar and Melchinger 2019). $N_2O$ inhibits polymerization of microtubules. Compared with colchicine and herbicide treatments, $N_2O$ treatment is easy to perform, and its efficiency is similar to that of colchicine (Kitamura et al. 2009). However, this treatment requires investment in a pressure chamber that can withstand high pressures, and this may increase the cost of initial establishment (Kato 2002; Molenaar et al. 2018). Furthermore, doubling at the immature-embryo stage by tissue culture using purple genetic markers (Chase 1949) and fluorescent markers (Zhao et al. 2013) could improve the doubling efficiency and reduce cost and time (Du et al. 2010). As the two markers came from the male parent, the embryos that lack purple color or fluorescent signals are haploid; otherwise, they are diploid (Fig. 2). The selected haploid embryos are put into N6 culture media for carrying out the subsequent doubling process with the chemical reagents (Chase 1949; Zhao et al. 2013; Chen et al. 2016).

## Emerging applications for haploid induction

Genome editing based on haploid induction (HI-Edit) is a new technique that has been in use in recent years. Recently, Wang et al. created a haploid inducer line containing CRISPR/Cas9 proteins designed to edit the *ZmLG1* gene. When this inducer line was used to hybridize an excellent maize inbred line, genome-edited haploids could be generated under any background. Since homozygous DH lines can be produced in two generations, this technique can bypass the intense and long backcross process and realize the molecular design of specific loci of commercial elite inbred lines. Moreover, since multiple gRNAs can be stacked into a CRISPR/Cas9 vector, this technique allows multiple gene loci to be edited simultaneously; thus, it can be used to aggregate several favorable alleles in an excellent background in a short time. In addition to the advantages listed above, genome-editing techniques are also suitable for lines that are resilient to transformation (Kelliher et al. 2019; Wang et al. 2019a).

Heterosis has been widely applied in agriculture to improve the productivity and adaptability of crops (Birchler et al. 2003; Schnable and Springer 2013). However, heterosis could not be maintained in offspring due to the chromosome recombination events during sexual reproduction, and the production of hybrid seeds requires consuming lots of labor, material, and financial resources at each year. Therefore, the fixation of crop heterosis will simplify the process of seed production and has important economic benefits. Notably, synthetic apomixis has been proposed as a way to fix the heterosis of $F_1$ hybrid crop varieties (Spillane et al. 2004).

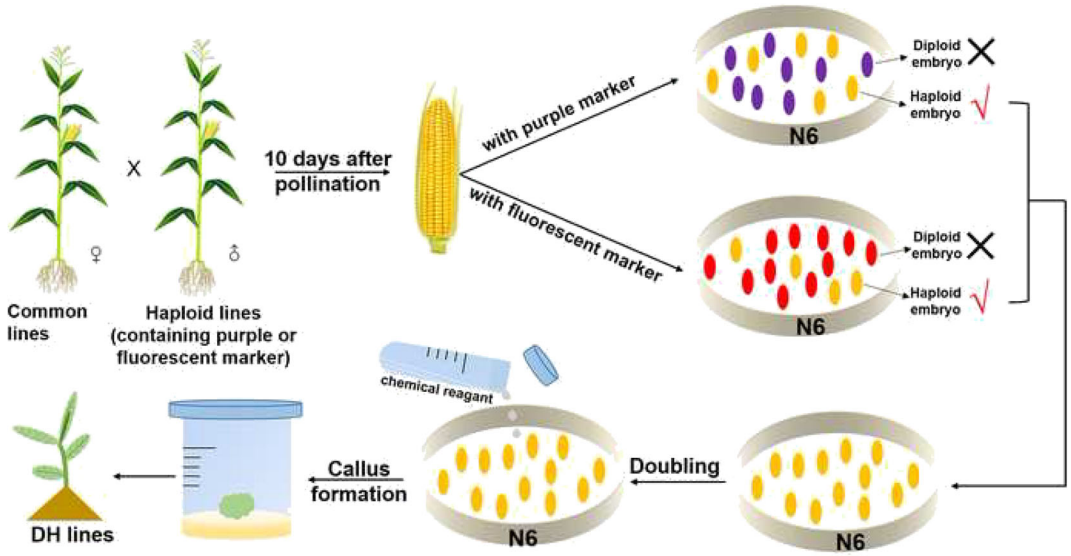


**Fig. 2** Model of the rapid breeding of DH lines by the use of inducer lines containing purple markers or fluorescent markers. The embryos that lack purple color or fluorescent signals are haploid. They are selected to put into N6 culture media for carrying out the subsequent doubling process with the chemical reagents. N6: short for N6 culture media

Apomixis refers to a kind of asexual reproductions in which seeds are produced in the absence of fusion of male and female gametes. Apomixis offers the possibility for direct production of hybrid seeds, which will further speed up the breeding process. To achieve the goal of apomixis, bypassing meiosis and fertilization are necessary to directly form embryos and produce seeds (Khanday et al. 2019). Combining the mutations of three genes *PAIR1*, *REC8*, and *OSD1* that mediate crucial meiotic processes resulted in the creation of a genotype named *MiMe* (mitosis instead of meiosis), in which meiosis is replaced by a mitosis-like division, resulting in the production of male and female clonal diploid gametes, as has been performed in *Arabidopsis* and rice (*Oryza sativa*) (d'Erfurth et al. 2009; Mieulet et al. 2016; Khanday et al. 2019). Furthermore, given that the mutation of the haploid induction gene *MTL/NAD/ZmPLA* can trigger haploid induction, a team at the Chinese Academy of Agricultural Sciences established a new apomixis system involving the *MTL* induction gene in rice. Four endogenous genes, *PAIR1*, *REC8*, *OSD1*, and *MTL*, were simultaneously knocked out to obtain diploid progenies with parental genotypes (Wang et al. 2019b). Additionally, genome-editing technology is now available for several crop species, and the function of *PAIR1*, *REC8*, *OSD1*, and *MTL* is conserved, so we anticipate that this method can readily be applied to crop species in which generation of $F_1$ hybrids is not currently commercially viable.

## Future perspectives

At present, although the haploid technique has been extensively used in commercialized breeding, the following four aspects remain to be improved. First, high-frequency haploid inducer lines containing excellent agronomic traits should be selected. Second, the effectiveness and accuracy of haploid identification should be improved by integrating multiple valid markers into the new inducer lines. Third, a highly efficient doubling system should be established by combining doubling rate-related QTL mapping and gene-editing technique. Finally, the time need for DH line production should be reduced by utilizing tissue culture and selection markers. In the future, resolving the technical challenges of the above aspects will promote haploid induction technique that could be more widely used in modern maize breeding. Furthermore, the use of haploid technique has been extended to other monocotyledon and dicotyledonous crop species, such as wheat (Liu et al. 2020), rice (Yao

et al. 2018), and *Arabidopsis* (Zhong et al. 2020), which will greatly improve the breeding efficiency of various plant species.

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