Doubled-Haploid Technology in Maize (*Zea mays* L.) and Its Practical Implications in Modern Agriculture

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Indu, Vijay Kamal Meena, Ranjit Saroj, Manoj Kumar Patel, Devender Sharma, Subhash Chand, Rajat Chaudhary, Rajesh Kumar Singhal, Reena Rani, and Amit Dadheech

Abstract

Maize (*Zea mays* L.) is the most important cereal crop in the world, consumed directly and indirectly. Doubled-haploid (DH) technology in maize has emerged as a promising tool for accelerating the development of completely homozygous lines in a much shorter time than conventional breeding methods. The breeding cycle is shortened and genetic gain is enhanced using the rapid doubled-haploid line generation method. Haploids are created mainly using traditional techniques, such as in vitro and in planta processes, and are then transformed into doubled haploids either naturally or through chemical means. The recent developments in

Indu · R. K. Singhal
Crop Improvement Division, ICAR-Indian Grassland and Fodder Research Institute, Jhansi, India

V. K. Meena
Agriculture Research Sub-Station (Sumerpur), Agriculture University, Jodhpur, India

R. Saroj · M. K. Patel · R. Chaudhary
Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

D. Sharma
Crop Improvement Division, ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora, Uttarakhand, India

S. Chand (✉)
Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

AICRP on Forage Crops and Utilization, ICAR-Indian Grassland and Fodder Research Institute, Jhansi, India

R. Rani
Division of Plant Improvement and Pest Management, ICAR-Central Arid Zone Research Institute, Jodhpur, India

A. Dadheech
Department of Plant Breeding and Genetics, RCA, MPUA & T, Udaipur, India

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understanding the genetic and molecular mechanisms of doubled haploidy have opened new avenues for precise genetic improvement in a shorter time. Marker-assisted breeding can be combined with doubled haploidy to fix favorable alleles for a variety of traits in a single DH line. Additionally, the method can be employed for reverse breeding, CMS line development, and uncovering the genetic diversity found in untapped germplasm and landraces. The future of DH breeding is bright since reliable DH production techniques are available and marker-assisted technologies are being more closely incorporated.

Keywords

Doubled haploid · Haploid inducer · Maize · Genetic gain · Induction rate

6.1 Introduction

Maize (*Zea mays* L.) is the third most important cereal crop after wheat and rice which is grown for its versatile use for food, feed, and industrial products. It fulfills the primary calorie requirement in many developing and developed nations. Demand for maize worldwide would be increased by over 50% from 558 million tons in 1995 to 837 million tons by 2020 (Pingali and Pandey 2001). The demand for maize in developing countries alone was projected to rise from 282 million tons in 1995 to 504 million tons by 2020 (IFPRI 2000).

It can grow successfully in tropical, subtropical, and temperate agroclimatic situations anywhere in the world due to its greater genetic variability. Development of fully homozygous inbred lines is the prerequisite for maize hybrid breeding programs. It was established that the primary goal of the maize breeder is to identify the best hybrid combination of parents in a maize population to grow seed corn that results in a good harvest (Shull 1908). In modern agriculture, farmers can grow two types of maize varieties: open-pollinated varieties (OPV) and hybrids. Since the development and popularization of single cross hybrids, the maize improvement programs are mainly focused on developing high-yielding single cross maize hybrids. Single cross maize hybrids are derived from two genetically diverse homozygous inbred lines. The inbred lines generally developed through conventional breeding techniques require more time and resources. This results in nearly homozygous inbred lines after six to ten generations of selfing (Odiyo et al. 2014). The availability of haploid inducers (maternal/paternal) made it possible to generate a completely homozygous (100%) inbred line in just two seasons. This saves time and resources for developing the maize inbred lines. The maternal haploid inducers and paternal haploid inducers have different induction rates.

It all began with identifying and developing naturally existing haploid lines in maize (Chase 1969). The study of Coe (1959), who used the haploid inducer "Stock 6" to produce haploids in maize, represents the main advancement in haploid breeding of maize. Maternal and paternal inducers are the two main categories of

haploid inducers. Paternal haploids employ haploid inducers as female parents, while maternal haploids use haploid inducers as pollen parents. In earlier investigations, the gene *ig1* (indeterminate gametophyte 1) was discovered to be a trigger for paternal haploid induction (Kermicle 1969; Evans 2007). However, the paternal haploid induction method is the least recommended by the researchers for maize breeding programs due to the low frequency of haploid induction (Kermicle 1994) and inheritance of cytoplasm from inducer line in haploids (Kermicle 1973). Maternal haploids, in contrast, inherit their cytoplasm and nucleus from the same female parent, making this approach superior to paternal haploid induction. With time, this approach has been improved because of the discovery of temperate inducers (WS14, MHI, PHI, CAUHOI, and RWS) with a higher haploid induction rate than Stock 6 (Wu et al. 2014), which have been extensively employed in maize breeding programs. Tropically adapted haploid inducer lines (TAILs) with high induction rates have also been developed for tropical regions where temperate inducers produce inferior outcomes. Various breeding efforts have been made in the last two decades to develop the haploid inducer lines with a greater induction rate. CIMMYT has developed the second-generation haploid inducer lines (CIM2GTAILS) with a higher haploid induction rate.

In the first season of the doubled-haploid technology, haploidy is induced in diploid maize plants, which results in the progeny's chromosomal pairs being reduced to single chromosomes. The haploid chromosome set is duplicated in the second season using a specific chromosomal doubling process (mainly colchicine), which entails making copies of each chromosome to produce pairs of identical chromosomes. The outcome is a diploid maize plant, often known as a "doubled-haploid (DH)" plant, because in each pair of chromosomes, one chromosome is a copy of the other chromosome and the plant has homozygosity levels of up to 100% (Fig. 6.1). Guha and Maheshwari (1964) presented anther culture method to produce haploids in a lab environment for the first time. Niizeki and Oono both created rice haploids in 1968. Since over 250 species have used DH technologies, creating DH lines from heterozygous material is not very time-consuming.

DH lines display the complete genetic variability at the beginning of the selection program, simplifying the screening of outstanding genotypes. As we know, greater genetic variance leads to high heritability of genotypes per se; testcross evaluations improve this accuracy; therefore, purely 100% homozygosity suggests that no remaining heterozygosity is hiding the genotype performance, thus assuring that line selection can be achieved earlier. DHs have more per se performance for morpho-agronomical characters because more selection is enforced during the haploidy level. When recessive alleles come in a homozygous state, it's pretty easy to chuck out recessive deleterious alleles effectively from germplasm pools because haploids cannot counteract their unfavorable impacts.

Resources that may be supplied for testcross evaluation are unavailable due to testing of multiple later generations. In short, DH technology allows breeders to examine more hybrid combinations in a shorter time, realizing maximum genetic gain per cycle, reducing developmental cost, and enhancing the efficiency of the

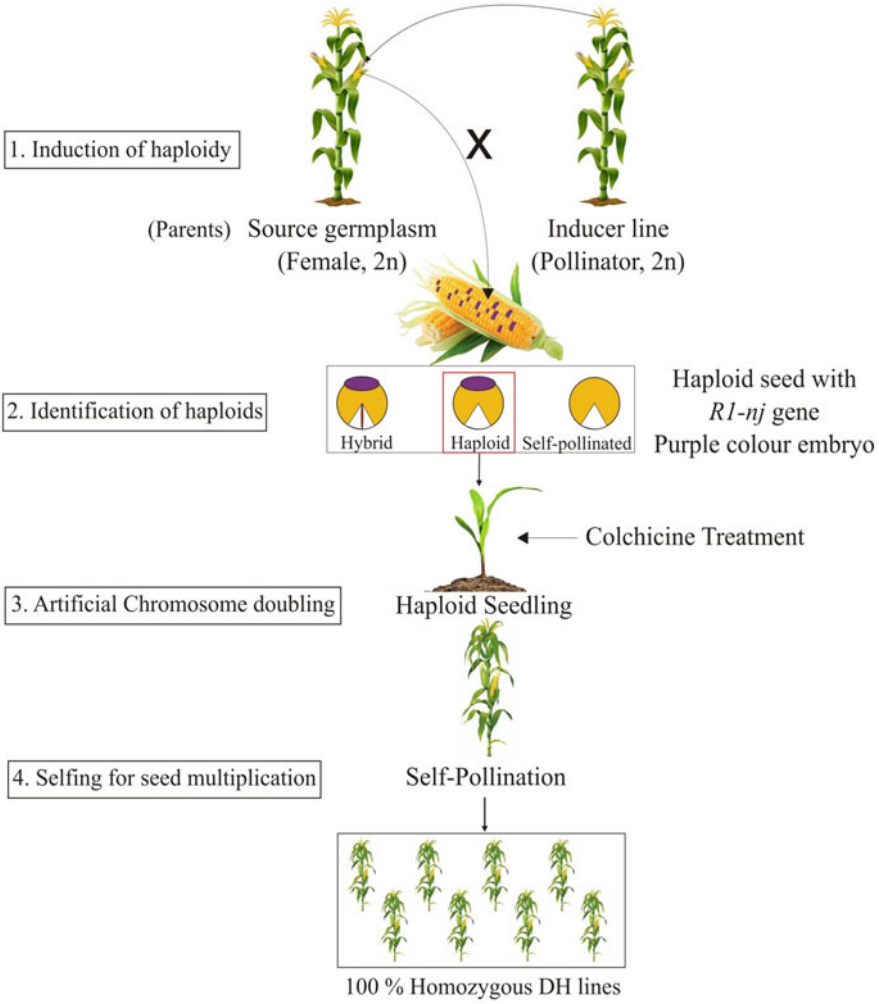

Fig. 6.1 An overview of steps involved in doubled-haploid technology

breeding program. The doubled-haploid approach may be regarded as the third most significant technology in a maize breeding program, after hybrid technology and off-season nurseries.

6.2 Haploid Generation

Production of haploids is a crucial stage in creating DH lines because it allows for the induction of haploidy both in vitro and in vivo. In vitro production of haploids requires aseptic conditions for the cultivation of anthers or microspores or female

(ovules) gametophytes to induce embryogenesis leading to the development of haploid plants. Generating in vitro haploids has not become a useful strategy in maize breeding because in vitro culture responsiveness in maize is entirely genotype dependent (Buter 1997; Tang et al. 2006).

Haploids can be generated in vivo by the following steps:

- Interspecific crossing in barley (*Hordeum vulgare*; by pollination with *H. bulbosum*) and wheat (by pollination with maize)
- Pollinating with the pollen that has been irradiated with heat or chemicals
- By crossing with a specific haploid inducer genotype (Chase 1952)

6.2.1 In Vivo-/Inducer-Based Approach

The proportion of haploids produced to the total induced cross progeny refers to the haploid induction rate (HIR) of the inducer employed. It is used to develop doubled haploids in maize. Haploid seed induction rate of inducer lines is 8–12%. Using the inducer as female will yield paternal haploids, while employing the inducer as male will produce maternal haploids. Table 6.1 mentions various maternal haploid inducer lines developed at different international institutions.

The DH technology adoption process in tropical countries is not as much as in temperate countries due to lacking of inducer lines having high HIR and wider adaptability. Molecular markers are being recently used for development of inducer

Table 6.1 List of maternal haploid inducer lines developed at various international institutions

Inducer line	Characteristics	HIR (%)	Researchers
Stock-6	Population of self-pollinated progeny	~3.2	Coe 1959
Wisconsin-23	Parental haploid inbred	3	Kermicle 1969
ZMK-1	Inducer population	6–8	Zabirova et al. (1996)
ZMK1U	Direct selection from ZMK1	11–13	Shatskaya (2010)
WS-14	W23gi × Stock-6	3–5	Lashermes and Beckert (1988)
RWS	WS14 × KEMS	7–9	Röber et al. (2005)
MHI	Carries *A1*, *B1*, *C1*, and *Rj-n1* alleles	7–9	Chalyk (1999)
PHIs	Four inducer lines (1–4)	10–15	Rotarenco et al. (2010)
CAU0I	High oil content	~3	Chen and Song (2003)
CAU5 and CAU079	High oil content	6–8	Xu et al. (2013)
UH600 and UH601	High oil content	~10	Melchinger et al. (2013, 2014)
TAIL	Tropical inducer line	~5–15	Prigge et al. (2012a, b)
CIM2GTAILS	Tropical inducer line	~5–15	Chaikam et al. (2018)

Table 6.2 A list of factors that affects the haploid induction rate

Factors	Particulars	HIR rate	References
Season	Winter	Higher	Kebede et al. (2011)
	Warmer	Higher	De La Fuente et al. (2018)
Crop	Barley (summer)	Higher	Pickering (1984), Pickering and Morgan (1985)
Silk age	Older	Higher	Chase (1969), Seaney (1954), Tyrnov (1997), Chase (1974)
Mode of haploid production	Hand pollination	Higher	Rotarenco (2002)
Donor genetic background	Flint Dent Flint × dent	Lower	Eder and Chalyk (2002)
	Hybrid derived from inbreds	Higher	De La Fuente et al. (2018)

lines (Dong et al. 2014). It was recently reported that genes encoded for pollen-specific phospholipase are necessary for producing seeds having haploid embryos. Prigge et al. (2012a, b) reported a gene *GRMZM2G471240* named *ZmPLA1*, located at locus QTL *qhir1*, showed 66% genetic variance in three populations obtained from a cross between inducer and normal germplasm line, and their results showed that HIR have epistatic genetic control. In haploid inducer development, single fertilization happens. A sperm cell fertilizes only the egg or central nuclei cell and forms a haploid embryo (Sarkar and Coe 1966). Maize impend double fertilization by starting the formation of a second pollen tube, which is fused with a second synergid cell. This mechanism is known as heterofertilization (Uliana Trentin et al. 2020). Sprague first reported the heterofertilization in maize in 1929, who stated that it occurs at an average of <2.0% (Sprague 1932). Inducer development is also affected by certain factors that are enlisted in Table 6.2.

The introduction of CRISPR-Cas9 construct into maize haploid inducer line having a transgene *CENTROMERIC HISTONE3* (CENH3) induces maternal and parental haploids (Ravi and Chan 2010). Kelliher et al. (2017) showed evidence that CRISPR-Cas9 can be combined with a different method of haploid induction efficiently and effectively into cultivars. Inbreds, hybrids and synthetics are developed in maize and could also be exploited for development of inducer lines.

6.3 Types of Inducer Parents

Most widely inbred lines are used as inducer parents to produce doubled-haploid progeny in plant breeding programs. However, hybrid inducers such as tropical climate-adapted hybrids developed by CIMMYT (Prasanna et al. 2012), RWS/RWK 76-a German hybrid (Flint-Garcia et al. 2003), and inducer population such as ZMK

1 (Shatskaya 2010) are also used. Each type of inducer parent has some advantages and disadvantages over each other.

6.3.1 Inbred as a Inducer

Globally inbred inducers are used as maternal haploid inducer parents due to their breeding true to type and uniformity, easy maintenance and multiplication, and rouging advantage of off types over all other inducers by only visual observations. Alleles for specific traits will be in homozygous state and would also be easy to incorporate them into new inducer inbred lines. If haploid sorting is done based on OC (oil content) value, then an inbred inducer will be best because there will be no classification error as if hybrid and composite inducer lines are used. The major limitations are that inbreeding depression tends to reduce hybrid vigor, changing plant morphological behavior, increases the susceptibility to major and minor diseases and insects pests, low seed setting, and fertility of pollens for a long spell. They show weaker performance in the isolation field to produce many haploids. However, they show better ergonomics when hand pollination is used in induction nurseries for limited seed production.

6.3.2 Hybrid as a Inducer

Hybrids used as inducers, being heterotic in nature, tend to produce larger tassels and have abundant fertile pollen and tolerance to diseases and insects. However, due to its gametophytic nature, HIR does not show hybrid vigor (Prasanna et al. 2012). The major challenges are that trait of interest must be in a homozygous state and to achieve it, both the parents must be in a homozygous state for the desired trait; otherwise, heterozygosity will tend to generate variability in the haploid progeny which makes them unsuitable for accurate screening and identification. Hybrid inducer lines need to create and maintain a separate genetic pool and spatial and temporal isolation for inbred maintenance and hybrid seed production. Hybrid inducer lines are much taller than inbred and synthetics, making them lodging susceptible, which is one of the key challenges, and unfit for areas where high wind speed prevails. Qualitative trait such as *R1-nj* is easy to incorporate, while quantitative trait such as OC is difficult, challenging, and time-consuming to incorporate in hybrid inducer at homozygous condition.

6.3.3 Synthetic as a Inducer

Synthetic inducers contain the desirable traits of both inbreds and hybrids. Synthetic inducer lines also show some extent of hybrid vigor over inbreds, but less than hybrid inducers, and the extent of vigor depends on the genetic dissimilarity between crossable genotypes. They are easier to develop and maintain if inbreds are used as parents. They produce fertile pollen for a long time spell due to more genetic

variability than inbreds and hybrids. These lines are not as heterotic as hybrids, thus producing less amount of pollens and comparatively more susceptible to diseases and insects. These lines must be reproduced at a periodic and regular time interval to maintain their vigor and desired trait level, which was jeopardized due to natural contamination and drift. A major challenge is the fixation of desirable marker traits in the developed population when more parents are involved in genesis.

6.4 Development of New Maternal Inducer Inbred Lines

Inducer inbred lines were extensively developed for temperate climatic conditions. But these inducer lines were not well eco-adapted to tropical conditions and showed poor agronomic performance under appropriate management practices. Thus, there is a separate need to develop new well-adapted inducer lines with good agronomic performance in tropical environments by using a robust tropical breeding program

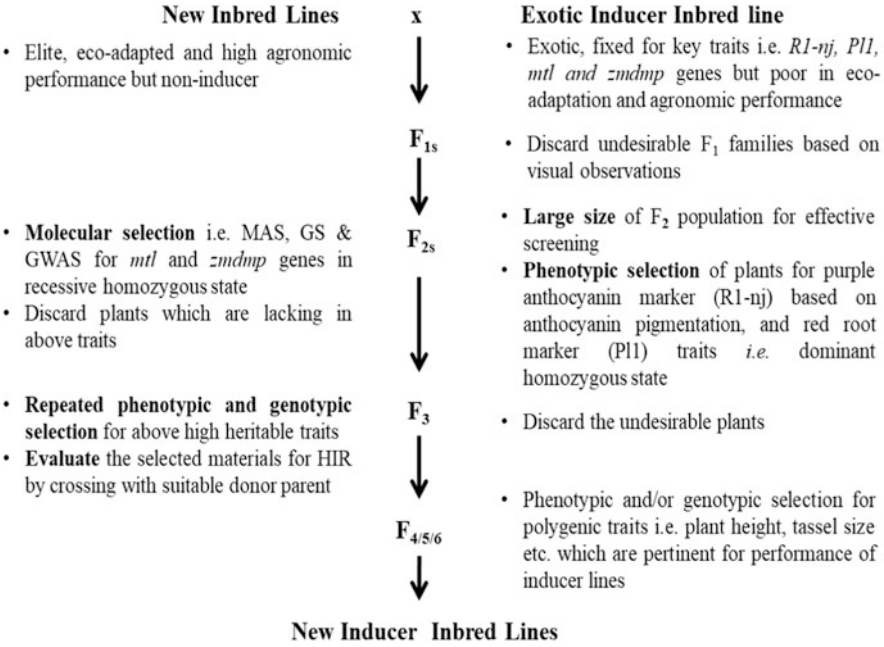

Fig. 6.2 Development of new tropical maternal haploid inbred lines by using exotic-cum-non-adapted and poor agronomic performance of temperate inducer inbred lines. Crossing between elite-non-inducer inbreds and exotic inducer inbred tend to produce F_1 families. Selection for moderate and high heritable traits such as purple embryo pigmentation (PEP), red root traits, and *mtl*, i.e., disrupts maternal haploid induction at an early generation of selfing (F_2 and F_3), while for low heritable traits such as resistance to some disease and yield, the selection is desirable in F_4 generation onward. The combination of phenotypic selection (PS) with genomic selection (GS) and genome-wide association studies (GWAS) accelerates the breeding progress, reduces the breeding cycle, and, ultimately, increases the genetic gain of desirable traits

(Fig. 6.2). Exotic inducer lines are fixed, i.e., homozygous state, with wide differential morphological marker genes such as *R1-nj* (purple embryo marker), *Pl1* (red root marker), *mtl* (maternal haploid induction), and *zmdmp* (increases the haploid induction rate of inducers). These exotic inducer lines are used as pollen sources, while elite or advanced well adapted and good in agronomic performance inbred lines are used as seed parents under the crossing program. A large number of F_1 families must be produced to avoid the loss of good genotypic material in the subsequent generations. Discard F_1 families which are undesirable for inducer lines. Selected F_1 progenies are self-pollinated to produce a large number of F_2 populations to obtain a desirable number of genotypes (~0.4%) with fixation of the above-mentioned genes (Uliana Trentin et al. 2020). Phenotypic selection (PS) can be made for the *R1-nj* trait, while marker aided selection (MAS) for *Pl1*, *mtl*, and *zmdmp* for their fixation in F_3 generation. In the subsequent generations, i.e., F_4 and onward, selection must be made for polygenic traits such as plant height, tassel length, pollen production and duration, haploid induction rate, lodging tolerance, and seed set.

Genomic selection (GS) has been used to improve traits essential for inducers. Nowadays, the DH breeding program combines GS to achieve maximum genetic gain. Through the integration of GS at a haploid level during haploid inducer line development, we can select only superior haploids through individual haploid genotyping for self-pollination, reducing the time and size of the population to be selfed. GS is based on prediction accuracies, and analysis is done by using genotypic and phenotypic data. Higher prediction accuracy tends to create a more accurate and precise selection for trait of interest. The most challenging is evaluating HIR for inducer lines which are complex, time-consuming, and labor-intensive. There must be a high seed production to evaluate accurate HIR. Human error is greater while separating the haploid seed from a mixture of selfed diploid and crossed diploid seeds. Haploid seeds are selected based on the expression of *R1-nj* gene (purple embryo pigmentation) in the embryo. Its expression also depends on factors such as environmental conditions (Prigge et al. 2011), seed morphology, and inhibitor gene from the donor parents (Paz-Ares et al. 1990). Thus, analyzing the HIR rate large sample size and the number of people involved might be time-consuming but will be most effective. Newly developed inducer lines can be effectively used for haploid seed production in tropical areas of the world.

6.5 Steps Involved in Doubled-Haploid Production Technology

6.5.1 Step 1: Detection of Putative Maize Haploid Seeds

The cross between normal germplasm and inducer inbred line generally produces three types of seeds: hybrid, haploid, and self/outcross. We can visually distinguish these seeds through an effective phenotypic marker system. Inducers carry a dominant gene, *R1-nj*, which can be used as an embryo- or endosperm-specific marker

gene, which induces purple coloration of the scutellum and the aleurone of seeds. The endosperm and embryo of normal maize plant are triploid and diploid, respectively, because they are aroused from fusion of two female polar nuclei with one male sperm cell and the fusion of the egg cell with the remaining sperm cell. Therefore, as the purple *R1-nj*-encoded coloration is dominantly inherited, only seeds of the haploid embryo will have a nonpigmented scutellum, while seeds with diploid embryos have purple-colored scutellum. In line with the before, scutellum pigmentation helps differentiate haploid and diploid seeds, whereas aleurone pigmentation helps to categorize haploid and diploid seeds from the outcrosses (without pigmentation) (Khulbe et al. 2022). Another phenotypic marker involves the Pl1 gene in which hybrid plant roots show red coloration, whereas haploid plant roots remain white. The mutant carrying recessive morphological traits such as liguleless or glossy appearances on leaves is the most authenticate method of identification of haploids. Tester for liguleless and glossy traits has been widely used to examine HIR during genetics-cytological studies, development of inducer, and maintenance activities. At the molecular level, through marker-assisted selection (MAS), we can identify the haploids by fixing the genes like *R1-nj* (purple colored embryo), *Pl1* (red root marker), *mtl*, and *zmdmp* in the adopted inducer inbred lines. Recently authors also stated that oil content of seeds can also be used in haploid seed selection. We have summarized some trait-specific genes important to haploid inducers and are helpful in distinguishing haploid seeds from diploids with their mode of gene action in Table 6.3.

6.5.2 Step 2. From Haploids to Doubled Haploids via Duplication of Chromosomes

In vivo production of maize doubled haploids involves artificial chromosome doubling as most haploid plants are sterile due to disrupted gamete formation. Therefore, doubling the haploid chromosomes is required for the seed set and maintenance of the genotype, so self-pollination can occur in doubled-haploid plant. In maize, the most common integral part of doubled-haploid standard protocol is colchicine, an alkaloid extracted from meadow saffron (*Colchicum autumnale* L.) that inhibits spindle fiber formation during mitotic division (Prigge et al. 2012a, b). Chromosome doubling through colchicine is the most promising and economic method as it has the most success rate; on the other hand, it is hazardous also. Trained persons are required for its handling, personal care, storage, and proper disposal after its use. The steps for chromosome doubling make the doubled-haploid technology expensive for its extensive use in developing countries.

Altogether, these constraints underline the necessity of replacing the colchicines with other alternative methods to spontaneously enhance chromosomal doubling. The treatment of haploids with nitrous oxide also observed anti-microtubule effects (Kato 2006). Cycloalkane is also reported as chromosomal doubling agent but it has not been adopted on large scale and limited information on its success rate is available (Cori Cui et al. 2013). To further have an alternative approach for

Table 6.3 List of genes/QTLs important for haploid induction and discrimination between haploid and diploid seeds

Genes/ QTLs	Trait	Genetic control	Gene action	Desirability	References
R1-nj	Purple embryo marker	Monogenic	Dominant	At seed stage haploid selection	Chase and Nanda (1965)
Pl1	Red root marker	Monogenic	Dominant	At seedling stage haploid selection	Emerson (1921)
B1 and Pl1	Purple sheath, husk, and culm	Digenic	Dominant	Before flowering haploid selection	Chandler et al. (1989)
mtl/nld/ zmpla 1, zmdmp	Haploid induction in maternal inducer	Monogenic	Recessive	Required for haploid embryo formation	Kelliher et al. (2017), Liu et al. (2017), Gilles et al. (2017), Zhong et al. (2019)
qhir 2–7, zmdmp	HIR of maternal inducers	Polygenic	Additive, dominant, and recessive	Efficiency determination in which haploid seeds are formed	Prigge et al. (2012a, b), Liu et al. (2015), Zhong et al. (2019), Chase (1947), Melchinger et al. (2014)
ig1	HIR of paternal inducers	Monogenic	Recessive	Efficiency determination in which haploid seeds are formed	Kermicle (1969), Kindiger and Hamann (1993), Lashermes and Beckert (1988)
lec1, DGAT1-2, OBAP1, WRI1	Oil content	Polygenic	Mainly additive	Oil content can be used to differentiate between haploid and diploid seeds	Moreno-Gonzalez et al. (1975), Berke and Rocheford (1995), Laurie et al. (2004), Zhang et al. (2008), Moose et al. (2004), Shen et al. (2010), Cook et al. (2012), López-ribera et al. (2014)

chromosomal doubling, Melchinger et al. (2015) used two phytohormones [amiprophos-methyl (APM) and pronamid] in their experiment in different ratios to treat the maize haploid seedling. They reached almost the same result as colchicine without risk of toxicity and suggested that pronamid at optimum dose is as good as colchicine for chromosomal doubling. A recent review suggests that detecting quantitative trait loci (QTLs) inducing spontaneous haploid genome doubling (SHGD) can be introgressed into the genome of the source germplasm by crossing

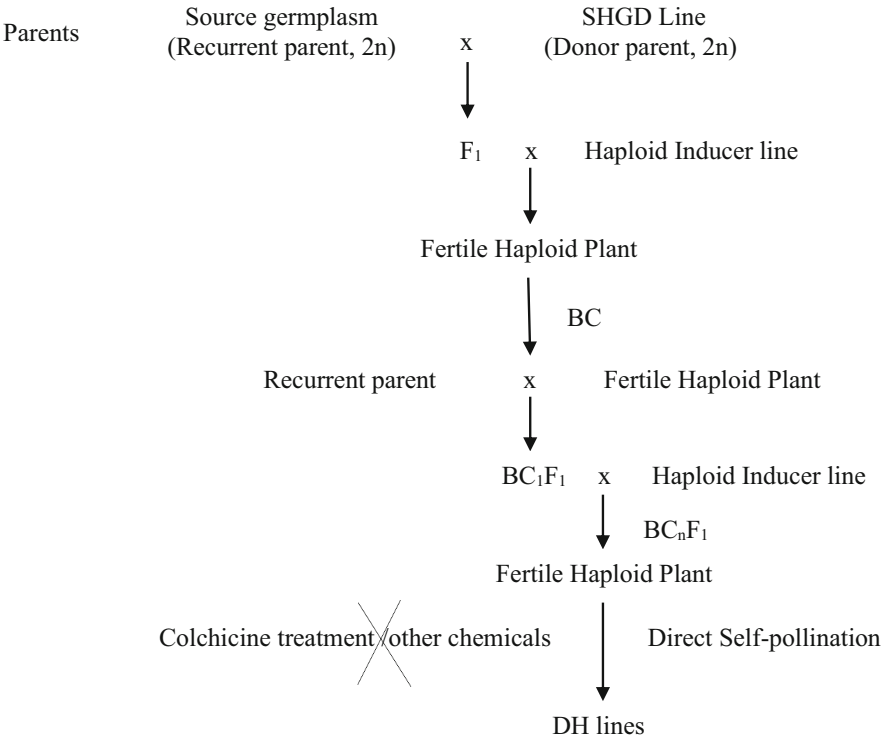

Fig. 6.3 Schematic representation of a breeding procedure for introgression of SHGD into non-SHGD source germplasm line and showing the haploid plants can directly be selfed without undergoing any chemical chromosomal doubling treatment

it with the donor SHGD line and their F_1 crossed with a haploid inducer line. These haploid seeds are repeatedly backcrossed to recurrent parents up to the desired number of times. The end progeny will have induced SHGD in its genome, and no chemical treatment is necessary for chromosomal doubling. These backcrossed introgressed SHGD-induced progenies can directly be selfed to produce DH lines (Boerman et al. 2020). It has been observed that SHGD chromosomal doubling increased from 5 to 50% and explained that epistatic gene interactions were present for SHGD, which could be exploited instead of artificial chromosomal doubling that ranges from 10 to 30% (Molenaar et al. 2019) (Fig. 6.3).

6.5.3 Step 3. Self-Pollination and Genetic Nature of D_1 DH Population

Plants treated with colchicine are called as D_o. Selfing of D_o plants will produce D_1 seeds. The D_1 consists of newly developed completely homozygous DH inbred

lines. Many D_o plants produce a limited number of seeds, as low as one. Just 3–5% of all haploid plants of a genotype will develop into DH lines. It has been reported that the genetic variance of a DH population is greater compared to segregating F_n populations obtained from the same parental cross (Seitz 2005). The more homozygous and homogenous nature of doubled haploid enhances the heritability compared to F_n segregating families. The genetic gain that increased through the use of the DH line can be calculated by using the following equation:

$$G_C = \frac{ih^2\sigma p}{t}$$

where i is the selection differential, h^2 is the narrow sense heritability of the selected trait (s), σp is the phenotypic standard deviation, and t is the time taken per breeding cycle (Boerman et al. 2020). DH population exhibits only additive genetic variance because of homozygosity at all loci and reflects higher covariance than any other population. The use of the DH population increases the genetic gain due to only additive genetic variance, which parallelly increases the response to selection, positively increases the heritability, and ultimately allows greater repeatability, through which environmental variation can be reduced by increasing replications.

6.6 Utilization of Doubled Haploids in Various Maize Breeding Programs

1. Geiger and Gordillo (2009) conducted an experiment by using maize doubled-haploid technology and suggested that the use of doubled haploids (DH) can be routinely used in maize (*Zea mays* L.). If off-season nurseries are available, two testcross generation evaluations can take place in only 4 years through developing one cycle DH line. When three breeding steps, including recombination, haploid induction, and DH plant development, are completed in a single year, then the duration of the cycle can be reduced to 3 years. Genome-wide marker-assisted selection can be incorporated effectively into DH line-based breeding technologies.
2. Smith et al. (2008) have suggested that DH progeny inherit a major portion from parental chromosomes. Third-generation DH progeny were selected that were more than 90% similar to one of the parents. They suggested that DH technology allows taking up the genome of a commercial hybrid already present in the domain. The study showed that the DH population has the largest area because it extends utmost toward extremes of parents' values. The study also conveyed that the DH population is more effective and efficient than the RIL and F_2 population in accessing the parental genotype to the utmost level.
3. Wu et al. (2014) used the inducer line CAU5 to pollinate a mapping population made up of 186 $F_{2:3}$ family lines developed from spanning Zheng58 and Chang7-2 and then choose the haploid kernels using *R1-nj* kernel markers to address the maternal genetic contribution to haploid formation. To find QTLs relating to

haploid inducibility, they created an $F_{2:3}$ population. On chromosomes 1 and 3, two quantitative trait loci (QTLs), *qmhir1* and *qmhir2*, were found which are involved in the maternal genetics of haploid induction.

4. Odiyo et al. (2014) experimented with 160 DH testcross hybrids and five checks. The material was evaluated under two locations; one was well watered and the other was at a drought location. Their combined analysis showed that the best 20 hybrids expressed better performance for grain yield and other agronomical characters of maize than the checks. The top ten DH testcross hybrids yielded 16% higher than the best check. While under drought location, the top ten DH yielded 62% higher than the best check. According to these findings, maize hybrids developed using DH lines had comparable grain yields and acceptable agronomic features to commercial hybrids produced using traditional pedigree techniques.
5. In 2006, Mayor and Bernardo (2009) examined 430 DH testcross lines in many environments, and marker-trait connections for grain yield, moisture, plant integrity, and staying green were found. The best DH lines in the initial mapping population were then intercrossed after three rounds of marker-assisted recurrent selection (MARS), performed from the F_2 of the original cross. They also chose the top DH lines for 2006 (Phen-1) and 2007 based on testcross phenotypic scores (Phen-2). In this study, Phen-1 came from screening the DH testcrosses in just 1 year at eight different sites, whereas Phen-2 came from screening a better selection of DH test crosses in 2 years at 17 different locations. Researchers have hypothesized that the additional screening conditions employed in Phen-2 compared to Phen-1 would allow more accurate identification of better DH lines.
6. Mahuku et al. (2011) studied temperate inducers UH400 and RWS for induction of tropical source germplasm that includes landraces, OPVs, and single cross hybrids. The identification of haploid seed was done using a seed purple color marker controlled by the *R1-nj* (R-Navajo) gene. Crosses were made between CIMMYT advanced lines as females and inducer hybrids RWS × UH400 and RWS × RWK as pollinators, as well as backcrosses to both parents. HIR for the two temperate inducers was generally high and similar with results obtained in the temperate zone, indicating that they can be directly used in the tropical environment. The source germplasm showed a significant difference in HIR. That indicates that source germplasm is an important factor that contributes to different HIR in addition to the inducer. Therefore, the number of plants to be induced to obtain the desired number of DH lines differs for different source germplasm. Furthermore, the winter season had higher HIR, which shows that the environment plays one important factor in determining HIR; thus, the winter season was more suitable than the summer season for induction at Agua Fria, Mexico. This confers that DH technology can be initiated directly with the temperate inducers by pollinating a sufficient number of plants of source germplasm under suitable environmental conditions.
7. Georgeta and Cristea (2016) used Procera Haploid Inducers (PHI), which are highly suited to temperate temperature circumstances due to their high inducer

rate (HIR) and ample and high-quality pollen and excellent phenotype. To produce haploids and doubled-haploid parent lines through PHI, three synthetic populations (SP) from the most significant heterotic groupings were crossed. Twenty DH parent lines plus the four original parental lines that served as the study's controls made comprised the 24 lines in each trial. There were three experiments, one for every set of DH parental lines that were a part of the three synthetic populations. As shown, DH parent lines outperform parental line components in synthetic populations for all attributes studied. The traits associated with atmospheric heat tolerance, like anthesis-silking interval and prolificacy, showed the best results. From their research, it can be inferred that haploid technologies are characterized by complete homozygosity of doubled-haploid lines, phenotypic and genotypic uniformity of doubled-haploid and hybrids, and increased anthesis-silking interval. These traits reduce time and costs in maize breeding and significantly increase the efficiency of selection procedures.

8. Ryu et al. (2016) settled this technology in Korea to identify haploid-inducing factors and to develop temperate inbred lines for hybrid breeding. Haploid induction was done by using eight populations crossed with inducer line (TAILs) and through treatment with colchicine (0.04%), and 12-h chromosome doubling was done. The 11 inbred lines' doubled-haploid lines were selected. The average haploid induction rate was 4.1% when the inducer was crossed with three maize populations. They may significantly shorten the time required for line development and improve Korea's maize breeding research technique.

6.7 Application of Doubled Haploidy

6.7.1 Rapid Development of Homozygous Lines

The development of homozygous lines such as inbreds in any cross-pollinated crops is an important breeding objective. Conventional breeding techniques such as pedigree, bulk, SSD, and backcross methods require much more time to develop inbreds. Even off-season nurseries and shuttle breeding require several rounds of inbreeding to select a homozygous line (Tadesse et al. 2012). However, due to residual heterozygosity in cross-pollinated crops, complete homozygosity cannot be attained (Baenziger and Peterson 1992; Baenziger and DePauw 2009). Hence, to save the valuable time of breeders, doubled-haploid technique can be adopted to obtain a complete homozygous line in one or few generations. Doubled-haploid (DH) technique aids in rapid crop improvement by reducing several cycles of inbreeding to obtain a homozygous line (Tadesse et al. 2012). After obtaining a homozygous line, It can be utilized further in several ways, such as a new variety (in self-pollinated crops), as parent in a hybridization program, or as a mapping population in a gene/QTL mapping program.

6.7.2 Cytogenetic Studies

Doubled-haploid technique is useful in cytogenetic studies such as chromosomal pairing and production of aberrant chromosomal complements like monosomics, nullisomics, etc. Being univalent, haploids provide special opportunities to study pairing relationships among chromosomes. Using a modern biotechnological technique like plant tissue culture, the production of homozygous lines became easy by exploiting the haplo-diploidization system (Baenziger and DePauw 2009; Wu et al. 2012). In some crops, the DH technique has also developed chromosome substitution and chromosome addition lines.

6.7.3 Selection Breeding

The DH technique results in a complete homozygous line; consequently, it favors additive genetic variance that eventually increases selection efficiency. DHs also had a role in the recurrent selection; the superior DH of the first cycle can be used as a parent for hybridization in the next cycle; however, slow genetic improvement is expected using this technique due to frequent crossing, DH production, and selection (Tadesse et al. 2012). Using the DH technique, rapid crop improvement was observed in maize and barley (Seguí-Simarro 2015). DH technique is the third most important milestone in maize breeding after hybrid and off-season nurseries (Seitz 2005). It has also been used in crops like *Brassica*, wheat, barley, and rice (Dwivedi et al. 2015). Haploids having a single copy of the genome express deleterious recessive alleles and can eliminate them in early generations.

So, this technique permits a more effective assessment of the genetic diversity of landraces and open-pollinated varieties that could be hampered by heterogeneity and deleterious effect (Melchinger et al. 2018). Homozygous lines obtained from the DH technique could be grown in different environments as these lines have wider adaptability due to a broad genetic base.

6.7.4 Mutation Breeding

Mutation breeding is an important application of the DH technique (Zhu et al. 1993). In *Brassica* species, in vitro screening of herbicide-resistant mutants can be achieved through the DH technique (Beversdorf and Kott 1987). Further, recessive mutants can easily be recognized by DH techniques as compared to conventional breeding methods. In DH lines, the selection of mutants for quantitative traits became easy due to the fixation of mutation and desired recombinant (DePauw et al. 2011; Wu et al. 2012).

6.7.5 Production of Male or Female Plant

DHs could have applicability in producing male or female plant from dioecious crop species like asparagus, hemp etc., as haploids can be produced from both male and female gametes.

6.7.6 Mapping Quantitative Trait Loci (QTL)

DH lines have been used as mapping populations in molecular mapping program (Chauhan and Khurana 2011). These lines are non-segregating and hence can be used as perpetual mapping populations. These lines are free from residual heterozygosity; consequently, they are equally effective in self- and cross-pollinated crops. In barley, doubled-haploid lines are used in marker-assisted backcrossing program to select strip-resistant lines (Chen et al. 1994). DH technique produces a mapping population in a few generations, resulting in rapid gene identification compared to other mapping populations. Further, using this technique, landraces and biparental populations can be applied for genomic selection and association studies (Melchinger et al. 2018).

6.7.7 Stability of Agronomic Traits

Haploids of wheat/maize crosses are used for genetic studies and crop improvements (Amin et al. 2010). DHs being homozygous lines are genetically stable; therefore, introduced variance could be identifiable at any stage of the breeding program (Suenaga and Nakajima 1993). Rapid production of fixed lines using the DH technique helps in improving the stability of various agronomic traits.

6.7.8 Bulked Segregant Analysis (BSA)

BSA uses two extreme bulks to identify putatively linked makers. Selecting extreme types for a particular trait is difficult in segregating mapping populations like F_2 as it may involve both heterozygotes and homozygotes in bulks of the dominant allele. In contrast, perpetual mapping populations like DHs involve only homozygotes in bulk, which excludes the possibility of ambiguity in the experiment. The DH lines remove the heterozygosity and confirm the disease reaction and its testing can be repeated several times (Knox et al. 1998). The use of DHs in BSA has wider applicability in crops like rapeseed and barley.

6.7.9 Exchanging Cytoplasmic and Nuclear Genome

Haploids could be easily applicable in rapid development of different cytoplasmic and nuclear genome combinations by transferring nuclear genome into a heterologous cytoplasm. Alloplasmic lines are the best-suited example, which can be developed using haploid inducer lines. Further, cytoplasmic male sterility can be transferred in two generations using this approach.

6.7.10 Reverse Breeding

DH technique has an important application in reverse breeding. Reverse breeding inhibits the meiotic crossing over in F_1 generation and results in nonrecombinant parental gametes; further, using the DH technique, these parental gametes can be developed into doubled-haploid plants. Original hybrids can be obtained by crossing complementing DH lines assigned to different heterotic pools based on genetic diversity.

6.7.11 Application in Crop Improvement

Doubled-haploid technology can be utilized in crop improvement. The best instance of crop improvement using DH technology is maize, which was used to develop inbreds within a short period of time. According to the breeders' equation, the genetic gain is inversely proportional to the time required. Therefore, the genetic gain can be maximized by reducing the time needed for inbred development, which could be achieved by adopting DH technology. In maize, inbreds and hybrids have been produced in a short period (Prasanna et al. 2012). Doubled-haploid populations contain more desirable agronomic traits of interest. Smaller population size is required to obtain homozygous targeted genes in doubled-haploid populations compared to traditional F_2 populations. In DH populations, an increase in the target genes helps identify favorable genotypes that carry all or maximum desirable alleles of genes under consideration. Marker-assisted gene stacking in combination with DH populations could be the best alternative to target gene fixation (Que et al. 2010). Apart from maize, DH technology could also be used for genetic improvement of other economic crops where haploid production is easy.

6.7.12 Genetic Studies in Crops

DH lines have been successfully utilized in understanding the genetics of any crop species. Doubled haploids carry duplicated haploid genomes through a chromosomal doubling mechanism; as a result, recessive genes can be expressed in early generations. Hence, phenotypic evaluation of recessive traits can be easily performed using such populations. DHs are also helpful in identifying random

recessive mutants in the population. Further, using DHs, gene action of any quantitative trait can be estimated by the sample mean of genotypic variance (Choo 1981) or by developing different segregation generations involving selected DH lines as parents.

6.8 Limitation of Doubled Haploids

Haploids and doubled haploids have been technologically advanced, employing several approaches such as genotypic selection, alterations in the composition of growth media and its conditions, and modifications to the plant growth environments (Maluszynski et al. 1996, 2003). However, the transition phase of the gametophytic to the sporophytic system, its genesis, and morphogenesis are still blurred. In the past, countless efforts have been made to decode the genetic and molecular basis of doubled-haploid developments in plants (Kyo et al. 2003). For example, anther culture technique has been widely used to develop doubled-haploid plants, particularly species belonging to Brassicaceae, Poaceae, and Solanaceae; however, this technique has a very low success rate in the species, particularly *Glycine max* belonging to Fabaceae (Hu et al. 1996; Rodrigues et al. 2004).

In forest tree breeding, haploid production is difficult due to uncontrolled pollen donor sources. These tree species have a robust structure that might be crucial in other species for DH production (Palmer and Keller 1999). Therefore, for the production of DH in these species, the main focus should be on the isolation of flower buds or inflorescences and their pretreatments. Two major challenges have been reported with DH production in tree species: successive rate and efficacy of embryo formation and enlargement (Bueno and Manzanera 2003; Bueno et al. 2003) and missing callus formation during the direct embryogenesis phase from microspores that is needed for reducing the gametoclonal dissimilarities and provides stability for the embryo at the genetic level (Deutsch et al. 2004). But these types of variation might be beneficial for the isolation of different and unique genotypes. There are several missing links to vividly understand the process of initiation and development of embryogenic tissue from microspores.

In addition, DH production using microspores faces major challenges due to recalcitrant type of nature and genotypic variability at the species level (Zheng et al. 2003). Male sterility does not permit the production of DH using microspores in the species belonging to Cucurbitaceae, Liliaceae, and Chenopodiaceae families; however, gynogenesis might be the best option. The development of DH from gynogenesis also has a lot of limitations, such as genotype specificity, a very less rate of haploid production, a high level of restriction during tempted chromosome doubling, and reduced fertility (Alan et al. 2003). The chromosome elimination method has also been used for DH production, especially when both androgenesis and gynogenesis could not be exploited (Mujeeb-Kazi and Riera-Lizaraza 1996); however, this technique could be used only in monocots. In addition, there are a few challenges while using this technique. For example, embryo development is regulated by pollen-contributing genotype, and the exact mode of chromosome elimination is

also unknown. Therefore, robust in vitro culture techniques such as embryo rescue and efficient chromosome doubling approaches are required for speeding up the DH production in crop species.

6.9 Conclusion

In experiments, the hybrids developed in maize by exploiting DH lines can give high corn yield and acceptable agro-morphological traits that are as good as hybrids developed by conventional breeding approaches. Hence, the elite DH lines could be used in hybrid maize breeding programs for high corn yield and tolerance to different biotic and abiotic stresses, particularly for drought and heat. Further, DH technology shortens the breeding cycle and increases genetic gain. The amalgamation of molecular or morphological markers with DH technology in breeding programs has different challenges in following the IPR issues under Plant Variety Protection regimes.

6.10 Future Prospectus

As previously mentioned, DH technology has many advantages over conventional breeding methods. In maize, it has modernized the breeding programs as the cost of investment in producing completely homozygous lines is less and these lines could be used for hybrid development and deployment for other trait improvements. However, sophisticated technology coupled with high technical skills is needed for producing DH lines and their effective implementation in breeding programs. Haploid production and chromosome doubling techniques are the main pillars required for DH technology. Although several decades of research have extensively been used for DH production, its genetic mechanism, in maize, for producing maternal haploids is still unclear. Conventional approaches for haploid genome duplication are toxic, labor-extensive, and cumbersome and use expensive reagents leading to restrictions for DH line development. However, haploid genome doubling technologies such as combining haploids and minichromosome approach could be of immediate use for accelerating DH production. In addition, we must search for novel markers that can easily detect the haploids with a very low false-positive rate.

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