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Topic Introduction

Doubled Haploid Technology: Opportunities and Challenges for the Rapid Generation of Maize Homozygous Lines

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Maize is used for multiple purposes, including food, feed, and energy production, and since transitioning to hybrid cultivars at around 1930, maize yield has significantly increased. This is largely due to hybrid vigor, which refers to the superior performance of the progeny from two unrelated inbred parents. Consequently, nearly all maize cultivars grown in the United States are hybrids. Hybrid breeding programs comprise two essential components; namely, inbred line development and hybrid production. Traditionally, developing inbred lines takes a long time, requiring six to 10 generations of self-pollination. The doubled haploid (DH) technology, however, accelerates this process, enabling the derivation of fully homozygous lines within two generations. DH technology is applicable in several crop species and has been most successful in maize due to in vivo maternal haploid induction. Here, we review the origins of the DH technology, and discuss advantages and challenges of the technology as well as applications of DH lines.

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INTRODUCTION

Maize serves as a food, feed, and energy crop, and is among the three most important crop species around the world, with 32% of the 2021 production residing in the United States (FAO 2021). Being a cross-pollinating species, maize has been cultivated as an open-pollinated variety, and transitioned to hybrid varieties at around 1930 (Bennetzen and Hake 2009). The first hybrids were double-cross hybrids involving three to four parent lines. This was necessary due to the lack of vigor and low seed production of the inbred parent lines. At that time, it was observed that hybrids yielded 15% more than the best open-pollinated variety, a difference attributed to improved management and heterosis (Duvick 1999). Heterosis—or hybrid vigor—refers to the superior performance of progeny from two unrelated parents that have been inbred for several generations (Shull 1952). Around 1965, nearly all maize cultivars grown in the United States were hybrids, and with advancements in inbred line performance, single-cross hybrids became more common.

Currently, hybrid breeding programs consist of two steps: inbred line development and hybrid production. In these programs, inbred lines are first developed within heterotic groups and then crossed between groups to obtain vigorous hybrids (Hallauer et al. 2010). Traditionally, inbred lines were generated by continued self-pollination: after five rounds of self-pollination, an individual plant's expected homozygosity reaches 97%. The problem, however, is that even when using off-

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season nurseries, conducting five generations of self-pollination will take 2–3 years to create almost homozygous inbred lines (Hallauer et al. 2010), making this a lengthy process.

Doubled haploid technology provides an attractive alternative, allowing the development of inbred lines in only two generations. This approach is efficient and reliable, and is routinely used in maize breeding programs worldwide (Chaikam et al. 2019). The goal of this approach is to derive completely homozygous lines from heterozygous plants. The heterozygous plants serve as donors; they provide the genome for the doubled haploid lines. Donors can be progenies of biparental crosses, backcrossing populations, or diverse populations, such as landraces (Bernardo 2009; Strigens et al. 2013). To create homozygous lines using the DH technology, the first step involves the development of haploid plants with a single set of chromosomes (n). The haploid plants are then treated with an artificial doubling agent, which inhibits spindle apparatus formation during mitosis, resulting in a doubled chromosome set in the nucleus. As these lines have the same haploid chromosome set doubled, they are called doubled haploids ($2n$). Because both chromosome sets are identical, doubled haploid lines are completely homozygous (Wan et al. 1991; Chaikam et al. 2019; Patial et al. 2019).

There are different ways to develop doubled haploids depending on the species, but generally, as mentioned above, the DH method involves two steps: generation of haploids and subsequent self-pollination after genome doubling. Haploid plants can be produced by in vivo or in vitro (anther or ovary culture) methods, or a combination thereof, and the preferred method varies by plant species. In wheat, for instance, haploids can be generated by anther culture or by a process known as wide hybridization. Wide hybridization—an in vivo method—involves pollinating wheat with millet or maize pollen. This results in haploid wheat embryos with nonfunctional endosperm, which then consequently need embryo rescue, an in vitro approach (Patial et al. 2019). In barley, haploids are developed similarly through anther or ovary culture, or via wide hybridization using *Hordeum bulbosum* as pollinator. For the "*bulbosum* method," barley is pollinated with *H. bulbosum*, and a few days after fertilization, the genome of *H. bulbosum* is naturally eliminated in the embryo and endosperm. Consequently, the embryo will be haploid and the endosperm will be diploid, resulting in a nonfunctional endosperm and nonviable embryo, making embryo rescue necessary (Patial et al. 2023). Therefore, in barley and wheat, in vivo methods (wide hybridization) are followed by in vitro work (embryo rescue).

Embryo rescue involves methods to turn otherwise nonviable or immature embryos into viable seedlings (Rogo et al. 2023). In angiosperms, double fertilization results in the formation of a diploid embryo and a triploid endosperm, which constitute the seeds. However, in wide crosses, such as those mentioned above of barley pollinated with its wild relative, the wild relative's chromosomes are eliminated during embryo development, leading to a haploid embryo and a diploid endosperm, which are typically nonviable. To overcome this, the haploid embryo is isolated and cultivated on a specialized nutrient medium, allowing the development of a viable haploid plant (Armenta-Medina et al. 2021). Generating haploids through in vitro cultivation is a complex process, demanding significant effort, time, and financial resources. Moreover, its success is contingent on the specific species and genotype involved (Dwivedi et al. 2015).

In maize, the in vitro step is not required for the generation of doubled haploids, which greatly simplifies the process. There are two theories regarding the in vivo haploid induction process in maize; namely, single fertilization and chromosome elimination in the embryo after double fertilization. Both mechanisms result in a functional triploid endosperm, thereby reducing seed abortion and eliminating the need for embryo rescue (Chaikam et al. 2019). Therefore, the in vivo DH technology is the primary method used in maize breeding programs for inbred line development. Using DH technology, fully homozygous inbred lines can be generated in two generations (achievable within a year), providing a major time advantage compared to the conventional way of deriving inbred lines via extensive self-pollination (Kleiber et al. 2012).

In this review, we discuss the advantages and challenges of the doubled haploid (DH) technology, with a focus on maize. We provide an overview of the origins of the DH technology, the key discoveries that made it possible, and the steps involved in its application. The discussion includes

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the mechanisms underlying the DH process and practical aspects of deriving homozygous lines using the DH technology.

ORIGINS OF THE DOUBLED HAPLOID TECHNOLOGY IN MAIZE

The origins of the DH technology can be traced back to the discovery of naturally occurring haploid seeds in maize, by Randolph and Stadler in 1929 (Randolph 1932). The potential applications of this phenomenon for creating homozygous inbred lines were grasped over time (Chase 1949), leading to the assessment of different genotypes for their haploid-inducing capabilities. One particular population, Stock 6, demonstrated higher inducing abilities and the capacity to enhance haploid induction rate (HIR) when backcrossed with various germplasms (Coe 1959). Ongoing breeding efforts have increased HIR from the initial 2%–3% to 12%–15% in modern haploid inducers (Rotarenco et al. 2010), and several genes associated with HIR in maize have been identified. Among these, a gene located on chromosome 1, *nld/mtl/zmpla1* (found by three independent research groups simultaneously, which is why it has three gene names) displays the largest impact, responsible for HIR ranging from 4% to 14%, depending on the genetic background (Gilles et al. 2017; Kelliher et al. 2017; Liu et al. 2017). Achieving that current HIR made the DH process become feasible, and consequently, DH technology is now widely adopted in breeding programs for creating homozygous lines (Geiger and Gordillo 2009; Dwivedi et al. 2015).

STEPS IN THE DOUBLED HAPLOID PROCESS IN MAIZE

In maize, creating homozygous lines using the DH technology involves four key steps: (1) inducing the donor germplasm, (2) identifying haploid seeds, (3) doubling of the genome, and (4) self-pollination of chimeric plants (Chaikam et al. 2019). For the first step, particular "inducer" genotypes, i.e., those with the ability to induce seeds with haploid embryos (n) when crossed to another plant, are used. Two theories have been proposed to explain the mechanism that leads to haploid embryos after pollinating with inducer plants: single fertilization and chromosome elimination in the embryo after double fertilization (Chaikam et al. 2019). If the seed-bearing parent possesses inducing ability, this process is called paternal haploid induction. Alternatively, if the pollen parent has inducing ability, this is referred to as maternal haploid induction (Trentin et al. 2020). Maternal inducers are more commonly used in maize due to their efficiency and convenience (De La Fuente et al. 2020). With maternal induction, the haploid seeds will be harvested from the female plant, which means they will have the cytoplasmic and the haploid nuclear genome of the female donor (Chaikam et al. 2019). When an inducer is crossed to a maize donor plant, progeny will segregate into diploid ($2n$) and haploid (n) classes.

Inducing the donor germplasm involves pollinating the donor germplasm with pollen from the inducer, which results in ~10%–15% haploid seeds, which require sorting from diploid seeds. Identification of haploid seeds is based on seed color markers, such as the purple embryo marker (*R1-nj*) developed by Nanda and Chase (1966). Once haploid seeds are identified, they are sorted and germinated in the greenhouse, and the resultant seedlings are treated with a doubling agent—typically colchicine—to double the genome, before being transplanted to the field. The resulting chimeric plants are self-pollinated, where possible, to obtain the first generation of doubled haploid plants (Chaikam et al. 2019).

APPLICATION AND ADVANTAGES OF THE DOUBLED HAPLOID TECHNOLOGY

DH lines are used in self-pollinating species, for line development, and also in outcrossing species, for the creation of inbred lines. In addition to the time advantage over conventional self-pollination, DH lines offer complete homozygosity and maximum genetic variance among DH lines (Geiger and

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Gordillo 2009; Boerman et al. 2020), which are beneficial characteristics for maize research and breeding. Reducing the time to derive fully homozygous inbred lines accelerates the breeding cycle in hybrid breeding programs, and having DH lines available earlier compared to conventional inbred lines allows for earlier testing and hybrid production (Dwivedi et al. 2015). DH lines further facilitate line maintenance in breeding programs and can be used to create mapping populations in research (Röber et al. 2005). Additional advantages include the fact that DH lines meet the criteria for obtaining Plant Variety Protection under the International Union for the Protection of New Varieties of Plants (UPOV), ensuring distinctiveness, uniformity within the progeny population, and stability over time, because they are fully homozygous (Geiger and Gordillo 2009; Kock 2021). DH technology can further facilitate the incorporation of new germplasm from diverse pools, such as landraces or synthetic populations, which helps broaden the genetic pool of maize germplasm in breeding programs (Strigens et al. 2013).

Another benefit of DH lines is their utilization in research, where DH lines have been part of QTL or association mapping studies in wheat (Prins et al. 2011), rice (Li et al. 2005), and maize (Vanous et al. 2018). Although recombinant inbred lines are typically used for QTL mapping studies, they need at least six generations of self-pollination to be created. Therefore, DH lines offer a distinctive advantage, requiring fewer generations to be derived (Seymour et al. 2012).

CHALLENGES OF THE DOUBLED HAPLOID TECHNOLOGY

While DH technology presents numerous advantages and is widely used in breeding programs, it also comes with limitations that require further research. These limitations are associated with the marker system, the haploid induction rate, and the haploid genome doubling step.

With a HIR of 15%, the majority of the resulting seeds are diploid, containing the genomes of both donor and inducer germplasm. Thus, markers are essential to distinguish between haploid and diploid seeds. Various screening systems exist, such as discrimination based on oil content (Melchinger et al. 2014), the red root marker (Chaikam et al. 2016), and the green fluorescence protein (GFP) marker (Yu and Birchler 2016). However, the *R1-nj* color marker is most commonly used (Melchinger et al. 2015). The dominant *R1-nj* allele induces anthocyanin coloration in the embryo and endosperm (Nanda and Chase 1966), and is present in most inducer lines (Melchinger et al. 2015). Donor lines, on the other hand, do not possess the *R1-nj* allele, and if fertilization of the embryo is not successful, the embryo will therefore be colorless. Because plants exhibit double fertilization, the endosperm that develops after the fusion of one sperm cell with two central cells of the female flower will develop as usual and will be colored due to the dominant *R1-nj* allele. If the fertilization of the embryo is successful and the other sperm cell fuses with the egg cell (Dresselhaus et al. 2016), both the embryo and the endosperm will be colored. In this way, haploid seeds (uncolored embryo with colored endosperm) can be distinguished from diploid seeds (colored embryo and colored endosperm). This method has been widely used and is quite effective but is time-consuming and error-prone in certain germplasms. This has thus prompted the exploration of automated methods, such as discrimination based on oil content with nondestructive measurements (NMR, NIRS) (Melchinger et al. 2015).

Considering budget constraints in breeding and research programs, a higher HIR is desirable to increase the efficiency of DH (Longin et al. 2007; De La Fuente et al. 2020). Indeed, breeding efforts have raised HIR from 2%–3% in Stock 6 to >15% in modern inducer lines (Rotarenco et al. 2010). Despite this significant success, efforts to increase HIR continue because, on average, 85% of seeds are diploid and thus discarded. Combining QTL and genes associated with HIR offers the potential for new inducers with increased HIR (Prigge et al. 2011). Higher induction rates would require fewer donor plants, because more haploid seeds per donor plant would be induced. Currently, taking all the steps of the DH process, the germination rate of maize, the pollination rate, and the average seed set into account, on average, two to three donor seeds are needed to create one DH line (see Doubled Haploid Facility, Iowa State University, https://www.doubledhaploid.biotech.iastate.edu/ordering-dh-production). Increasing HIR, however, also has its drawbacks, as inducers possess self-inducing

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ability, leading to induced haploid seeds when self-pollinated. This means that if inducer plants are self-pollinated for increase, ~15% of the seeds harvested will be haploid, which in this case is undesirable because inducer seeds are directly planted and those haploid plants will not shed pollen, making them unsuitable for induction crosses. Moreover, it has been observed that kernel abortion increases with increased HIR (Qu et al. 2020).

Another bottleneck is the use and efficiency of the colchicine treatment for genome doubling. Colchicine is a toxic chemical that requires careful handling, proper storage and disposal, and trained personnel (Melchinger et al. 2015). Colchicine inhibits microtubule formation during mitosis (Bartels and Hilton 1973), which leads to nuclei with doubled genomes. However, because the process of cell division in plants takes place asynchronously, not all nuclei will be affected by the colchicine, leading to chimeric plants with both diploid and haploid cells (Wan et al. 1989). For chimeric plants to shed pollen and produce silks, the cells that develop into male and female reproductive organs must be doubled. Typically, haploid plants are able to produce silks, but their pollen production is limited (De La Fuente et al. 2020). Only 16%–42% of treated plants will be shedding pollen in the field and can therefore be self-pollinated for the final step of the process (Eder and Chalyk 2002).

The last step in the DH approach is the self-pollination of the chimeric plants, which will lead to doubled haploid seeds. Seeds from each harvested ear represent a new DH line, where all cells are in the diploid state (Chaikam et al. 2019). Nevertheless, this step is often challenging because some of the colchicine-treated plants will be pollen-sterile or their anthesis silking interval will not match. This is one reason for the reduced efficiency of the colchicine-mediated doubling step (Eder and Chalyk 2002).

The challenge of pollen-sterile plants could be addressed through spontaneous haploid genome doubling (SHGD), which restores male fertility without using colchicine as a doubling agent. Genotypes with SHGD, exhibiting >50% haploid male fertility, have been identified (De La Fuente et al. 2020). Implementing SHGD in donor populations could enhance genome doubling efficiency, reducing time and costs related to not only germinating the haploid seeds in the greenhouse, but also treating them with colchicine and transplanting the seedlings to the field. Indeed, exposure to this toxic chemical is avoided (Boerman et al. 2020). Incorporating SHGD into elite breeding populations, however, may have consequences, such as potential linkage drag of unfavorable genes or repercussions from fixing the SHGD QTL region in all hybrids (Foster et al. 2024). As such, further research is needed before implementing SHGD in breeding populations.

Despite all of these limitations, and as discussed above, the in vivo DH method in maize offers numerous advantages compared to conventional inbred line development (Geiger and Gordillo 2009). The in vivo DH technology in maize is simpler than both in vivo and in vitro methods used in other species, mostly because it does not require embryo rescue (Dwivedi et al. 2015). Moreover, through several advancements, this method has been widely adopted in breeding programs worldwide (Chaikam et al. 2019). In addition, this approach has set the stage for the development of new technologies. Indeed, haploid-induced (HI) editing using maize pollen from haploid inducers has been proposed for facilitating gene editing in maize and wheat. In this approach, the paternal genome with the editing machinery is eliminated in the haploid seed, and after self-pollinating treated haploid plants, *trans*-gene-free inbred lines can be obtained (Kelliher et al. 2019).

As part of this collection, we provide a protocol that outlines how to derive DH maize plants. The protocol uses maternal haploid inducers carrying the *R1-nj* allele for haploid discrimination, and colchicine for the doubling step (Grüning et al. 2024). The workflow described involves the generation and subsequent identification (screening) of haploid seeds, colchicine treatment of haploid seedlings, and self-pollination of treated plants.

CLOSING REMARKS

DH technology is an important part of research and breeding programs, providing numerous advantages and a basis for further research. Compared to the process in other species such as barley, wheat,

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or rice, in vivo haploid induction in maize is efficient and relatively straightforward. DH technology is unique in deriving completely homozygous inbred lines, both significantly reducing the time to obtain them compared to conventional inbred line development, and providing material for research applications.

Ongoing research focuses on enhancing the efficiency of the DH technology in maize through improved haploid induction rates, advanced marker systems, and optimized doubling techniques. DH lines prove valuable for QTL or association studies, and are important in breeding programs not only as an alternative to traditional inbred line development but also to diversify the genetic basis of breeding programs by extracting DH lines from landraces or synthetic populations.

COMPETING INTEREST STATEMENT

The authors declare no competing interests.

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AUTHOR CONTRIBUTIONS

Conceptualization: V.K.G. and U.K.F. Funding acquisition: T.L. Project administration: T.L. Supervision: T.L. and U.K.F. Visualization: V.K.G. Writing—original draft: V.K.G. Writing—review and editing: T.L. and U.K.F.

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